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The importance of H-RAS 81T-C polymorphism in cellular behaviour

Dissertação apresentada à Universidade de Coimbra para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Biologia Celular e Molecular, realizada sob a orientação científica da Doutora Patrícia Castro (Instituto de Patologia e Imunologia Molecular da Universidade do Porto) e supervisão da Professora Doutora Maria da Graça Vale (Faculdade de Ciências e Tecnologia da Universidade de Coimbra).

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Table of Contents

Abbreviations

2PG	2-phospho-D-glycerate
AChE-R	Enhancing enzymatic activity of enolase
AKT	Cellular homologue of transforming v-AKT
APC/C	Anaphase promoting complex/cyclosome
ARF	ADP ribosylation factors
BAX	Bcl-2-associated X protein
BrDU	Bromodeoxyuridine
BUB1	Budding uninhibited by benzimidazoles 1 homolog
BUB1B	Budding uninhibited by benzimidazoles 1 homolog beta (gene)
BUB3	Budding uninhibited by benzimidazoles 3 homolog
CDC20	Cell division cycle 20 homolog (S. cerevisae)
CENP-H	Centromere protein H
CIN	Chromosomal instability
DNA	Deoxyribonucleic acid
ERK	Extracellular signal-regulated kinase
FOXO1	Forkhead box O1
FUS/TLS	Fused in Sarcoma / Translated in liposarcoma
GAP	GTPase-activating proteins
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GDP	Guanosine diphosphate
GEF	Guanine exchange factor
GTP	Guanosine triphosphate
H-RAS	Harvey rat sarcoma
HEK293	Human embryonic kidney 293
hnRNP A1	Heterogeneous nuclear ribonucleoprotein A1

HVR	Hypervariable region
ICMT	Isoprenyl cysteine carboxymethyl transferase
IDX	Intron D exon
K-RAS	Kirsten rat sarcoma
MAD2	Mitotic arrest deficient 2
MAPK	Mitogen-activated protein kinase
MDM2	Murine double minute 2
N-RAS	Neuroblastoma rat sarcoma
NSE	Neuron-specific enolase
PCR	Polymerase chain reaction
PEP	Phosphoenolpyruvato
RAB	RAS-related GTP-binding protein
RAN	RAS-related Nuclear protein
RAS	Rat sarcoma
rasISS1	RAS intronic silencer sequence
REC1	RAS converting enzyme
RHO	Ras homolog gene family
RNA	Ribonucleic acid
mRNA	Messenger ribonucleic acid
RT	Reverse transcriptase
TUNEL	Terminal deoxynucleotidyl transferase dUTP nick end labelling

Resumo

A aneuploidia é um dos fenótipos mais comuns do cancro, estando presente na maioria dos tumores sólidos e cancros hematológicos (revisto em Cahill *et al*., 1999, e em Weaver e Cleveland 2006). A aneuploidia tem sido associada a invasividade e a estádios avançados (Rabinovitch *et al*., 1989; Mendelin *et al*., 1999; Veltman *et al*., 2000), podendo ser utilizada como ferramenta no prognóstico (Friedlander *et al*., 1984; Alcaraz *et al*., 1994; Risques *et al*., 2003) e na resposta à terapia (Watanabe *et al*., 2001), em várias neoplasias. O oncogene RAS tem sido associado à instabilidade genómica, através da indução da amplificação génica (Wani *et al*., 1994), de conteúdo cromossómico anormal (Denko *et al*., 1994; Saavedra *et al*., 1999) e da segregação incorrecta dos cromossomas em linhas celulares (Hagag *et al*., 1990; Saavedra *et al*., 1999), através da via MAPK (Saavedra *et al*., 1999) e da indução da amplificação dos centrossomas (Saavedra *et al*., 2000; Zeng *et al*., 2010). Recentemente foi demonstrado que o polimorfismo 81T-C do H-RAS está associado a alterações na ploidia em neoplasias foliculares da tiróide e em bócios nodulares, resultando num aumento da aneuploidia em lesões tumorais de pacientes que possuem o alelo C (Castro *et al*., 2006).

O principal objectivo deste trabalho foi esclarecer a importância do polimorfismo 81T-C do oncogene H-RAS no fenótipo tumorigénico no que se refere à ocorrência de aneuploidia. Para alcançar este objectivo estudámos: o(s) efeito(s) do polimorfismo 81T-C do H-RAS na expressão das isoformas (p21 e p19); o(s) efeito(s) deste polimorfismo no conteúdo cromossómico das células; e o efeito do polimorfismo no fenótipo tumoral.

Um dos resultados mais interessantes consistiu na demonstração da existência de uma associação do polimorfismo 81T-C do H-RAS com a modificação do splicing alternativo e, consequentemente, a alteração da expressão das isoformas p21H-RAS e p19H-RAS. Verificamos ainda que as células que sobre-expressam o H-RAS

contendo o alelo C na posição 81 apresentam um aumento do seu potencial tumorigénico, com aumento do crescimento celular, diminuição da apoptose e aumento da migração celular, em comparação com as células que sobre-expressam o H-RAS contendo o alelo T nessa posição. As células que sobre-expressam o H-RAS contendo o alelo C na posição 81 mostram, também, um aumento do número de cromossomas, bem como uma maior instabilidade cromossómica.

Palavras-chave: Oncogene, H-RAS, instabilidade cromossómica, aneuploidia, splicing alternativo, centrossomas.

Abstract

Aneuploidy is one of the hallmarks of cancer, being present in most solid tumours and hematological cancers (reviewed in Cahill *et al*., 1999, and in Weaver and Cleveland 2006). Aneuploidy is associated with high-grade and invasive tumours (Rabinovitch *et al*., 1989; Mendelin *et al*., 1999; Veltman *et al*., 2000), and it can be used as a tool for the prediction of patient prognosis (Friedlander *et al*., 1984; Alcaraz *et al*., 1994; Risques *et al*., 2003) and therapy responses for several neoplasias (Watanabe *et al*., 2001). The RAS oncogene has been associated, in cancer cell lines, with genomic instability by inducing gene amplification (Wani *et al*., 1994), generating aberrant chromosomal content (Denko *et al*., 1994; Saavedra *et al*., 1999) and inducing chromosome missegregation (Hagag *et al*., 1990; Saavedra *et al*., 1999), through the MAPK pathway (Saavedra *et al*., 1999), and through induction of centrosome amplification (Saavedra *et al*., 2000; Zeng *et al*., 2010). Recently, our group showed that the H-RAS 81T-C polymorphism is associated with alterations in the ploidy status in follicular thyroid tumours and in nodules of goitres, resulting in the increase of the aneuploidization of these tumours (Castro *et al*., 2006).

The main goal of this work was to further clarify the importance of the H-RAS 81T-C polymorphism in the H-RAS role on aneuploidy and tumourigenesis. In order to achieve this we decided: to study the effect(s) of the H-RAS 81T-C polymorphism in the expression of the H-RAS p19 and p21 isoforms; to assess the effect of this polymorphism, in cells aneuploidy; and to study the role of this process in the tumourigenesis.

We found the H-RAS 81T-C polymorphism to be associated with modifications in the alternative splicing and, consequently, with an alteration in the p21H-RAS and p19H-RAS isoform expression and their ratio. We demonstrated that the H-RAS C allele, in comparison with the T allele, is associated with an increase of the tumourigenic features, such as increased chromosomal instability, increased cell

growth, decreased apoptosis and increased cell migration.

Keywords: Oncogene, H-RAS, chromosomal instability, aneuploidy, alternative splicing, centrosomes.

Chapter 1 Introduction

1.1 – Tumourigenesis

Cancer can be defined as a tissue regulation disease, since it appears to be due to failure of the mechanisms that control cell growth and division, either by excess of proliferation and/or by lack of cell death. Hanahan and Weinberg (2000) defined six hallmarks of cancer: self-sufficiency in growth signals, insensitivity to anti-growth signals, evading apoptosis, sustained angiogenesis, limitless replicative potential and tissue invasion and metastasis. Later on, several authors have added other "hallmarks" to these, such as inflammation (reviewed in Colotta *et al*., 2009), metabolism (reviewed in Shaw 2006, and in Hsu and Sabatini 2008) and aneuploidy (reviewed in Weaver and Cleveland 2006). All these hallmarks are based on an altered expression or function of specific genes - tumour suppressor genes and proto-oncogenes - that ultimately will lead to abnormal cellular regulation. Proto-oncogenes are important genes in normal cell growth and development, which in their activated forms (by mutation, amplification or rearrangement), will lead to abnormal cell cycle progression and proliferation (reviewed in Nishimura and Sekiya 1987, in Anderson *et al*., 1992, and in Croce 2008). Tumour suppressor genes have the ability to promote cell cycle arrest and apoptosis, and, therefore, the loss or inactivation of these genes is common in cancer (reviewed in Hinds and Weinberg 1994, and in Macleod 2000).

1.2 – Chromosomal instability

Chromosomal instability (CIN) was considered to be a hallmark of cancer by several authors (reviewed in Cahill *et al*., 1999, and in Weaver and Cleveland 2006). CIN has been considered to be an adaptive response of cancer cells to the environment pressure (reviewed in Jallepalli and Lengauer 2001), and Nowell (1976) advanced that gain and loss of chromosome material is a process of diversification that

leads to the survival of the fittest clones. These authors apply the concept of population genetic diversity to the cellular context, which means that the more diverse they are the more likely they are to survive the constrains. The great majority of cancer cells are aneuploid and display dynamic karyotypic changes, including gain and/or loss of whole chromosomes - aneuploidy.

1.2.1 – Aneuploidy and cancer

Aneuploidy is considered to be a hallmark of cancer, since it is present in most solid and hematological malignancies (reviewed in Cahill *et al*., 1999, and in Weaver and Cleveland 2006) (Table I), being defined as a cellular state in which the cells present an abnormal number of chromosomes, due to loss or gain of chromosomes, that is not a multiple of the haploid number (reviewed in Yuen and Desai 2008). In a cancer cell, large genomic alterations affect the normal cell metabolism and function by amplification and/or elimination of chromosome segments containing particular genes and also by altering the transcription profiles of many genes (reviewed in FitzPatrick 2005). Cancers are autonomous cells defined by individual clonal karyotypes or stem lines much like individual species (reviewed in Sandberg 1990, and in Heim and Mitelman 1995; Mitelman database 2010).

Aneuploidy is known to be linked to the development of high-grade and invasive tumours (Rabinovitch *et al*., 1989; Mendelin *et al*., 1999; Veltman *et al*., 2000), it can be used as a tool for the prediction of patient prognosis in several neoplasias (Friedlander *et al*., 1984; Alcaraz *et al*., 1994; Risques *et al*., 2003) and to predict therapy responses (Watanabe *et al*., 2001).

It is important to acknowledge that tumours can be aneuploid but karyotypically stable, meaning that at some point of their growth, the tumours, after presenting unstable chromosome alterations, appear to be stable, probably because, by chance, they reach an optimum combination of imbalances (Roschke *et al*., 2002). It is toughly

that these alterations in the karyotype may follow a specific pattern (Heselmeyer *et al*., 1997) (Figure 1), being indicative of the tumour stage (Fujimaki *et al*., 1996; Katsura *et al*., 1996), metastatic potential (Aragane *et al*., 2001; Bockmuhl *et al*., 2002) and drug resistance (reviewed in Schimke 1984; Li *et al*., 2005; Nahi *et al*., 2008).

The role aneuploidy plays in tumourigenesis has been under discussion for decades. If aneuploidy has a causal role in the tumourigenic process or if it is just a product of the tumourigenesis, or both, remains unclear.

Table I - Analysis of chromosomal content in several solid and hematological tumours (reviewed in Weaver and Cleveland 2006)**.**

	Number of tumors that have not gained or lost chromosomes*	Number of aneuploid tumors with a near-diploid number of chromosomes (≤68)	Number of aneuploid tumors with a near- tetraploid number of chromosomes (≥69)
Solid tumors			
Astrocytoma, grade III–IV	10	228	62
Basal cell carcinoma	23	75	4
Breast cancer	31	140	29
Cervical cancer	4	51	29
Colon adenocarcinoma	1	124	19
Embryonal rhabdomyosarcoma	9	53	12
Hepatoblastoma	17	80	3
Leiomyosarcoma	7	68	34
Lung cancer	36	119	45
Malignant melanoma	30	138	31
Neuroblastoma	28	109	58
Osteosarcoma	6	86	59
Ovarian cancer	5	158	37
Prostate cancer	16	141	43
Retinoblastoma	10	111	1
Squamous cell carcinoma	12	149	39
Teratoma	3	166	31
Percent of solid tumors (n = 2780)	8.9%	71.8%	19.3%
Haematopoietic cancers			
Acute myeloid leukemia	88	207	3
Adult T-cell lymphoma/leukemia	21	224	8
B-prolymphocytic leukemia	20	72	1
Burkitt lymphoma/leukemia	86	75	2
Chronic myeloid leukemia	90	110	0
Follicular lymphoma	55	228	17
Hodgkins disease	26	129	77
Multiple myeloma	64	217	17
T-prolymphocytic leukemia	25	111	0
Percent of haematopoietic cancers (n = 1973)	24.1%	69.6%	6.3%
Percent of solid and haematopoietic cancers (n = 4753)	15.2%	70.9%	13.9%

* These cancer cells have 46 chromosomes containing translocations, inversions, deletions and/or additions but have not gained or lost entire chromosomes.

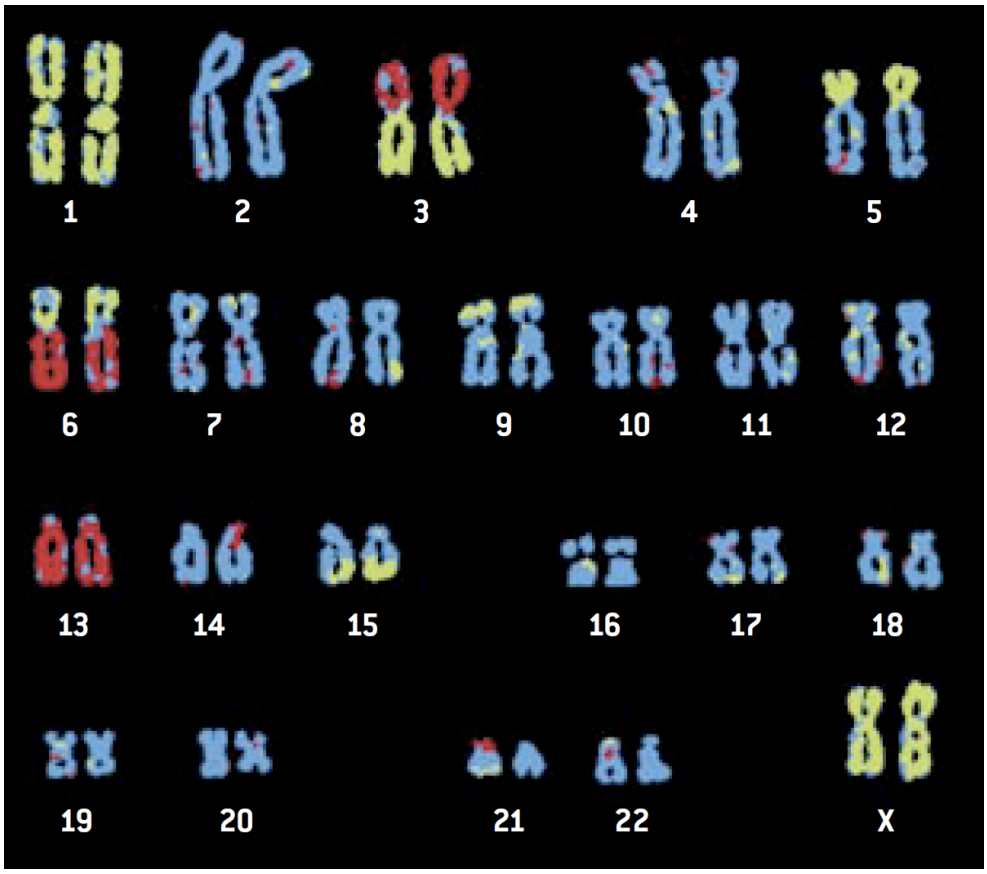


Figure 1- Consistent pattern of chromosome alterations in 30 cervical cancer patients (adapted from Heselmeyer *et al*., 1997). The red indicates loss of the region and the green indicates addition of genetic material.

The idea that aneuploidy may be the cause of cancer was first proposed around the end of the nineteenth century and the beginning of the twentieth century, by Hansemann and Boveri (Hansemann 1980; Boveri 1914). Hansemann initially observed the occurrence of uneven cell divisions in a small number of epithelial cancers (Hansemann 1890) and considered that the abnormal features presented by the tumour cells were caused by the abnormal chromosomal content of these cells (reviewed in Bignold *et al*., 2006). Around the same time, Boveri also observed unbalanced chromosome segregation in sea urchins dispermic eggs, due to the presence of multiple spindle poles (Boveri 1902). Boveri noticed that the some embryos had an abnormal development, that may led to the embryo death (Boveri

1914), which was also observed in *Drosophila melanosgaster* a few years later (Bridges 1921a; Bridges 1921b). Boveri also noticed that other embryos presented a cancer-like phenotype and advanced that the tumours may have a similar origin (Boveri 1914). This hypothesis was then disregarded with the identification of gene mutations, namely in oncogenes and tumour suppressor genes (Stehelin *et al*., 1976). However, recently, the hypothesis of aneuploidy as a cause of tumourigenesis has reappeared and, it has gained support with several experiments that explain several aspects of the tumourigenesis that may not be explained by the gene mutation hypothesis (reviewed in Duesberg *et al*., 2005, and in Duesberg 2007).

According to the gene mutation hypothesis, somatic mutations in specific genes (proto-genes and tumour suppressor genes), can lead to tumour development. In 1999, Hahn *et al* (Hahn *et al*., 1999) were able, for the first time, to transform normal human epithelial and human fibroblast cells into tumourigenic cells, by transfecting these cells with an immortalization gene and two oncogenic viruses homologous from oncogenes gathering proofs in favour of the gene mutation hypothesis (Hahn *et al*., 1999). As a response, Li *et al* (2000) asked the authors for these cells and observed that these transformed cells pass through a stage of pre-neoplastic aneuploidy, before becoming transformed (Li *et al*., 2000). Moreover, to achieve the neoplastic phenotype the cells had to undergo 60 population doublings, generating 10^{28} cells out of one, which would be equivalent to 10000 human bodies (Li *et al*., 2000).

The evidence that non-mutagenic carcinogens, such as asbestos, tar, mineral oils, naphthalene, polycyclic aromatic hydrocarbons, butter yellow, urethan, acrylamide, hormones, spindle blockers among others (Berenblum and Shubik 1949; reviewed in Burdette 1955, and in Oshimura and Barrett 1986), lead to the aneuploidization of the cells (Duesberg *et al*., 2000; Fabarius *et al*., 2002) is one of the arguments used by the defenders of the aneuploidy hypothesis.

The gene mutation hypothesis not only does not explain the appearance of a large number of mutations that leads to the development of cancer but also cannot

explain the fast alteration of the cancer cells phenotype, since the natural rate of mutations in humans is very low (reviewed in Loeb 1991, and in Duesberg et al. 2005).

The presence of aneuploidy in pre-neoplastic lesions, such as Barrett's oesophagus and ulcerative colitis (Levine *et al*., 1991a; Rabinovitch *et al*., 2001; Doak et al. 2003; Olaharski *et al*., 2006) is also a strong argument in favour of the aneuploidy hypothesis. Aneuploidy was also detected in Chinese hamsters cells treated with a carcinogenic agent, before their transformation (Duesberg *et al*., 2000; Fabarius *et al*., 2002). All of these data indicate that aneuploidy may be a necessary intermediate step in the formation of many human solid tumours (Li *et al*., 2000; reviewed in Duesberg *et al*., 2005).

Another line of evidence comes from patients with the mosaic variegated aneuploidy syndrome, a rare autosomal disorder in which patients are more prone to mitotic non-disjunction of the chromosomes, leading to high levels of aneuploidy, presenting an abnormal development and higher risk of cancer development (reviewed in Jacquemont *et al*., 2002, and in Ganmore *et al*., 2009). Studies with these patients have shown biallelic germline mutations of mitotic checkpoint genes of BUB1B, one missense mutation and one mutation that lead to a truncated protein, being both mutations present in the tumours (Hanks *et al*., 2004).

1.2.2 – Routes towards aneuploidy

1.2.2.1 – Tetraploidy as the origin of cancer

There are several ways and different hypotheses on how a cell becomes aneuploid. One of these hypotheses is that aneuploid cells pass through a previous tetraploid stage: this hypothesis has been supported by several reports (reviewed in Storchova and Kuffer 2008). Some tumours have a tetraploid or near-tetraploid DNA content even in early stages (Levine et al. 1991a; Levine *et al*., 1991b; Reid *et al*.,

1996), and aneuploid cells have extra centrosomes, which could indicate a previously aborted cell division (reviewed in Fukasawa 2005). The tetraploid cells can then start to lose chromosomes in each cell division.

Studies made in breast cancer and cervical cancer have suggested that the aneuploidy may have it is origin in the duplication of the whole genome and subsequently random loss of individual chromosomes (Dutrillaux *et al*., 1991; Verdoodt *et al*., 1994). Evidences supporting this hypothesis have also been observed in some pre-neoplastic lesion, such as Barrett's oesophagus and ulcerative colitis, since these lesions present high levels of tetraploid cells and aneuploid cells (Levine *et al*., 1991a; Rabinovitch *et al*., 2001; Doak *et al*., 2003; Olaharski *et al*., 2006).

The mechanisms involved in the whole genome duplication are yet unclear, even though many theories have emerged. The telomeres appear to play an important role in this process since telomerase negative immortalized cells are prone to develop tetraploid cell populations (der-Sarkissian *et al*., 2004). The cell fusion is another mechanism that can give rise to tetraploid cells; this event is a programmed step in some types of cells important for their differentiation and repair, such as skeletal muscle cells and osteoclasts (reviewed in Taylor 2003, and in Ishii and Saeki 2008). Nonetheless cell fusion also occurs during disease, like infections, and in cell culture, but the mechanism that lead to this outcome is unknown (Deaven and Kreizinger 1971; reviewed in Hernandez *et al*., 1996).

Mitotic failure may also lead to tetraploidy. The mitosis can be aborted due to several reasons - defects in DNA replication, loss of sister-chromatid cohesion, defects in mitotic spindle function and failure of cytokinesis (Minn *et al*., 1996; Lanni and Jacks 1998; Waizenegger *et al*., 2002). Normally, the mitotic checkpoint only produces a transient block in the progression of the cell cycle, until the stimuli disappears; but if the stimuli is persistent, the cells are able to slip the blockage and give rise to polyploid cells (Minn *et al*., 1996; Lanni and Jacks 1998). One known checkpoint protein involved in the cytokinesis failure, by being over-expressed is the AURORA A, which leads to

chromosomal instability (Meraldi *et al*., 2002). The over-expression of AURORA A was also associated with development of mammary tumour in mouse (Wang *et al*., 2006), which is consistent with the fact that aurora kinase A being over-expressed in the tumours (reviewed in Meraldi *et al*., 2004).

1.2.2.2 – Spindle assembly checkpoint failure

The spindle assembly checkpoint is a mechanism that prevents cells from undergoing through mitosis without having the chromosomes correctly aligned and attached to the mitotic spindle (reviewed in Rajagopalan and Lengauer 2004). This mechanism has the ability to delay the metaphase-anaphase transition, allowing the correction of putative mistakes, such as the correct attachment of the microtubules to the kinetochore. This checkpoint is mostly localized at the centromere and at the kinetochore, a complex protein structure where the microtubules attach to the chromosomes (reviewed in Yen and Kao 2005). The attachment of microtubules to the kinetochore will lead to the appearance of pulling tensions that will indicate if the chromatids are being correctly attached. One of the proteins responsible for regulating the attachment, through this mechanism, is the AURORA B/IPL1 kinase (IPL kinase is an homolog of aurora B in yeast), although is yet unclear the exact way how AURORA B will respond to the incorrect attachment (Hauf *et al*., 2003).

The metaphase-anaphase is highly regulated by the cell and only begins after the "wait anaphase signal" sent by the mitotic checkpoint is extinguished. This wait signal consists in the inactivation of the APC/C (anaphase promoting complex/cyclosome) complex, a large ubiquitin kinase (reviewed in Page and Hieter 1999), responsible for the securin degradation (Yanagida 2000). The securin on the other hand, is inhibiting the separin protease (responsible for the separation of the sister chromatids). When the securin is degraded, the separin becomes active and cleave the cohensins, until then they will keep the sister chromatids united (Cohen-Fix *et al*., 1996; Yamamoto *et*

al., 1996). The securin degradation will lead to the degradation of key proteins, necessary for the exit of the mitosis (Figure 2) (Sudakin *et al*., 1995). The APC/C complex is kept inactive by the highly regulated interaction of several proteins: MAD2 protein, which monitors the kinetochore occupancy (Waters *et al*., 1998) and inhibits of the ubiquitylation activity of the complex (Li *et al*., 1997; Fang *et al*., 1998; reviewed in Shah and Cleveland 2000, and in Gorbsky 2001); BUBR1-BUB3 complex, which inhibits the binding of the CDC20 to the APC/C complex, probably due to signals from the kinetochore (Abrieu *et al*., 2000; Tang *et al*., 2001).

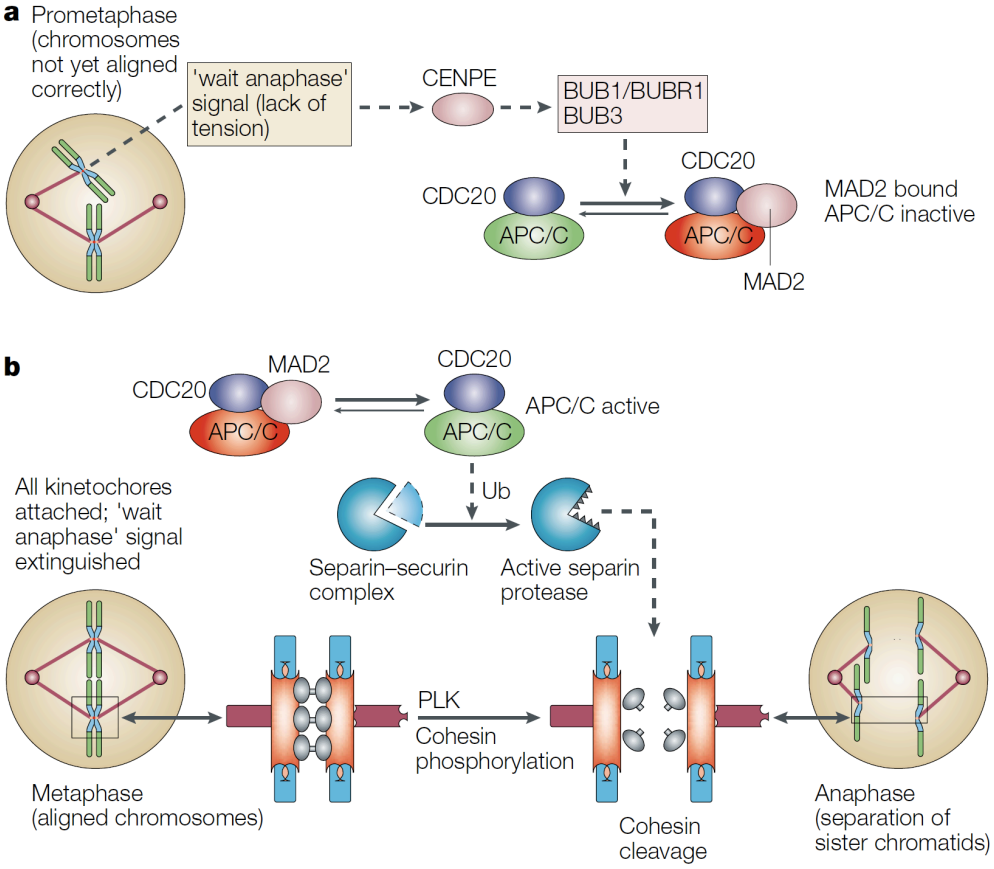


Figure 2 – Spindle assembly checkpoint (adapted from Jallepalli and Lengauer 2001). A) A wait anaphase signal is been generated from the unattached chromosomes, which leads to the binding of the MAD2 to the APC/C – CDC20 complex, inhibiting it. B) When the waiting signal is terminated, the MAD2 separate from the APC/, which becomes active. This

complex will then lead to the activation of the separin and the cleavage of the cohesins.

Due to the important role of these proteins in the cell cycle, and particularly, in chromosome segregation, the alteration of the levels of these proteins will cause aneuploidy. When the levels of MAD2 are reduced, the cells enter prematurely in anaphase and there will be chromosome loss (Michel *et al*., 2001). On the other hand, depletion of securin (which is an anaphase inhibitor) will lead to nondisjunction of the chromosomes, due to an impaired activation of the separin and to an inefficient cleavage of the cohensins (Jallepalli *et al*., 2001). Several studies in tumours and cancer cell lines have demonstrate that the expression of various proteins from the checkpoint are altered: securin was found over-expressed in NIH3T3 cell line and FTC133 (Zou *et al*., 1999; Honda *et al*., 2003; Yu *et al*., 2003; Solbach *et al*., 2004); mutations of BUB1 or BUB1B were found in a low percentage of colorectal tumours (Cahill *et al*., 1998); CENP-H was shown to be up-regulated in colorectal cancers (Tomonaga *et al*., 2003) and when transfected in diploid cell lines, it induce aneuploidy (Tomonaga *et al*., 2005). It is important to stress that, although several authors thoroughly searched for mutations in these mitotic checkpoint genes in human cancers, they are extremely rare, suggesting that the cells would not survive without proper function of these genes.

1.2.2.3 – Centrosome amplification

Centrosomes are microtubule-organizing centers, present in the animal cells, which are responsible for coordinating all microtubule-associated events, including their doublings, cell cycle regulation and the ultimately mitosis (reviewed in Nigg 2002). This cellular organelles are constituted by a pair of centrioles, fibers, pericentriolar

material and γ-tubulin complexes (reviewed in Lingle 2005); being the centrioles the centrosomal organizer (reviewed in Preble *et al*., 2000).

Centrosomes play an important role in the mitosis, not only in the assembly of the bipolar spindle, but also in the cytokinesis (reviewed in Hinchcliffe and Sluder 2001). Centrosome amplification may interfere with mitosis, due to the formation of multiple spindle poles, leading to chromosome missegregation (reviewed in Fukasawa 2005). Several authors reported that there can be clustering of centrosomes, whenever there are extra centrosomes, and that this is one cellular mechanism for dealing with the extra centrosomes. These cells can cluster the centrosomes, in a way that allows the formation of the bipolar spindle (Ring *et al*., 1982; reviewed in Nigg 2002).

The centrosome amplification may occur due to several mechanisms: defects in the centrosome cycle, (reviewed in Fukasawa 2005); cytokinesis failure, which not only leads to centrosome amplification, but also to tetraploidy (reviewed in Fukasawa 2005); the improper split of the centrioles, and the subsequently formation of the centrosome (Hut *et al*., 2003).

1.2.2.4 – The role of RAS proteins in aneuploidy

RAS oncogene has been shown to induce genomic instability - gene amplification (Wani *et al*., 1994), generation of aberrant chromosomal content (Denko *et al*., 1994; Saavedra *et al*., 1999) and chromosome missegregation (Hagag *et al*., 1990; Saavedra *et al*., 1999) in cancer cell lines, through the MAPK pathway (Saavedra *et al*., 1999). Previous work from our group showed that a polymorphism in the H-RAS gene - 81T-C polymorphism - was linked to alterations in the ploidy status in follicular thyroid tumours and in nodular goitres, being the C allele associated with patients having aneuploid tumours (Castro *et al*., 2006).

Recent studies have linked RAS oncogene to the induction of centrosome amplification and, consequently, genomic instability, in thyroid tumours and in

mammary epithelial cells (Saavedra *et al*., 2000; Zeng *et al*., 2010).

1.3 – The RAS family

The RAS proteins belong to the RAS superfamily of small guanosine triphosphates (GTPases), which comprise a large number of members divided in five families of proteins, based on their sequence and functional similarities: RHO, RAS, RAB, RAN and ARF (reviewed in Takai *et al*., 2001, and in Wennerberg *et al*., 2005). All the members of this superfamily are GTP-binding proteins, have an intrinsic GTPase activity, which can act as molecular switches, being the active form GTP-bounded, whereas the inactive form is GDP-bounded. They are regulated by several proteins such as guanine nucleotide exchange factor (GEFs), which control the switch of GDP for GTP and by GTPase-activating proteins (GAPs), that control the ability to hydrolyze GTP into GDP (reviewed in Lim *et al*., 1996, and in Wennerberg *et al*., 2005).

The RAS family has been under extensive research since three of the RAS genes were shown to possess oncogenic transforming proprieties - H-RAS, K-RAS and N-RAS (reviewed in Takai *et al*., 2001, and in Wennerberg *et al*., 2005). These genes are located in different chromosomes: the H-RAS gene is located in the short arm of chromosome 11 (11p15.5); the K-RAS gene is located in the short arm of chromosome 1 (1p13); the N-RAS gene is located in the short arm of chromosome 12 (12p12.1) (reviewed in Macaluso *et al*., 2002). Their gene structure and sequence are very similar, with 5 exons each, being the first non-codifying. These genes are ubiquitously expressed, although their levels of expression vary between different tissues (reviewed in Lowy and Willumsen 1993, and in Omerovic *et al*., 2007).

The RAS genes give rise to proteins that are highly homologous and conserve between them, with 21kDa, being generally designated as p21RAS (Parks and Scolnick 1977; Shih *et al*., 1979a; Shih *et al*., 1979b). The K-RAS and the H-RAS

genes are known to originate two different isoforms, through alternative splicing of their mRNA (reviewed in Malumbres and Pellicer 1998; Codony *et al*., 2001). The K-RAS gene is able to originate the K-RAS4B, the most common isoform, and the K-RAS4A - that has an extra exon (E4A), which leads to differences in their carboxy-terminal residues (reviewed in Malumbres and Pellicer 1998). The H-RAS gene can also originate two different proteins, the p21RAS and a smaller protein, with 19 kDa, designated as p19H-RAS (Cohen *et al*., 1989; Guil *et al*., 2003a).

1.3.1 – RAS genes history and relevance

RAS family was first identified in studies using rat sarcoma (RAS) induced by retroviruses. These studies started in 1964, when Jennifer Harvey used retroviruses from leukemic rats to induce sarcomas in rodents (Harvey 1964) and, in 1967, Kirsten isolated a different lineage of retroviruses (Kirsten and Mayer 1967). In 1974, Ed Scolnick and his associates established that the Harvey and Kirsten sarcoma viruses (Ha-MSV and Ki-MSV respectively) strains were the result of the recombination of retrovirus with sequences from the rat genome (Scolnick and Parks 1974), although the characterization of these sequences was only made in 1979 (Hager *et al*., 1979; Tsuchida and Uesugi 1981). Later it was established that these viruses were the result of the recombination of replication competent (helper) retrovirus, 30S RNA sequences of ancestral retrovirus and rat cellular genes (H-RAS for Ha-MSV and K-RAS for Ki-MSV) (Ellis *et al*., 1981). Afterwards, in 1983, a third transforming gene was identified in a neuroblastoma cell line, N-RAS gene (Shimizu *et al*., 1983), which was also shown to possess transformation potential (Hall *et al*., 1983).

Evolutionary evidence, such as the high degree of conservation of the genes, indicates that this family is indispensable for normal cellular functions (reviewed in Omerovic *et al*., 2007). In order to better understand the importance of the RAS genes and if each gene has a specific function or if they are truly redundant, several studies

with knockout mice to each RAS gene have been performed. The results of this experiments showed that the knockout mice for H-RAS and N-RAS or even double knockout mice for both these genes can survive (Esteban *et al*., 2001), but knockout mice for K-RAS didn't survive after mid-gestational age, due to several defects in the heart, liver and anemia (Johnson *et al*., 1997; Koera *et al*., 1997). Afterwards, the studies with knockouts for K-RAS shown that the only isoform essential for the survival of the mice is the K-RAS4B, whereas the isoform 4A is not essential (Plowman *et al*., 2003). Later, it was demonstrated, that introducing H-RAS sequence in K-RAS locus would allow the animals to survive and develop normally until the adulthood. This result shows that it is not the K-RAS expression by itself that is essential for the normal development of the mice since H-RAS, regulated by the regulatory sequences of K-RAS, is able to rescue the wild-type phenotype. These animals showed abnormalities only in the adulthood, at heart level, which indicates that K-RAS sequence is indeed required for a normal heart development and function (Potenza *et al*., 2005).

Surprisingly, recent studies showed that the K-RAS4A isoform promotes apoptosis and does not affect the spontaneous incidence of tumours, unlike the K-RAS4B isoform, that inhibits apoptosis and promotes the incidence of tumours (Plowman *et al*., 2006). These features appear to indicate that the 4A isoform has a tumour suppressor role, in contrast to the oncogenic role of the 4B isoform, which was demonstrated recently, with the deletion of the 4A exon in mice, after DMH treatment, the mice lacking the 4A isoform developed more adenomas, had more proliferation and showed a decrease in the apoptosis, in comparison with the control mice; they also present the activation of the MAPK kinase and AKT pathways, without the existence of K-RAS mutations (Luo *et al*., 2010). These data indicate that the two isoforms of the K-RAS oncogene have completely opposite functions.

1.3.2 – The RAS proteins post-translational modifications and localization

The H-RAS, K-RAS and N-RAS genes present a 90%-100% sequence homology between them, except in the hypervariable region (HVR), where the similarities are inferior to 15% (Figure 3). This region contains the information responsible for the trafficking and membrane binding of these proteins and it terminates with a CAAX motif (C stands for Cysteine, A for aphylatic aminoacid, X for any aminoacid) that undergoes several post-translational modifications, directing the proteins to the plasma membrane (reviewed in Prior and Hancock 2001, and in Omerovic *et al*., 2007).

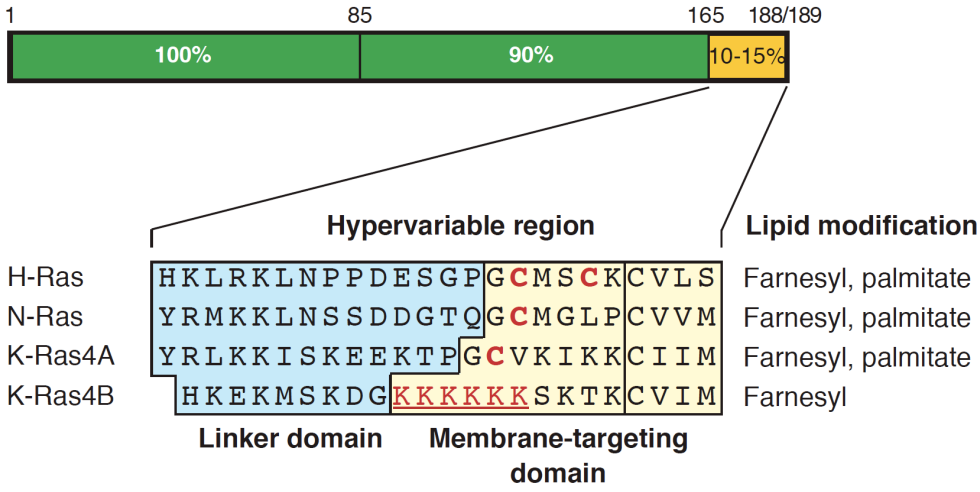


Figure 3 - Different RAS protein sequences, demonstrating the low homology of the hypervariable region (adapted from Prior and Hancock 2001).

The first modification of the CAAX motif is the addition of farnesyl isoprenoid lipid to the cysteine in the C terminal (prenylation reaction), by farnesyl protein transferase, which facilitates the binding to the endoplasmic reticulum. This reaction is followed by the cleavage of the AAX motif by RAS converting enzyme (REC1). Afterwards, the farnesylated cysteine is methylated by isoprenyl cysteine carboxymethyl transferase

(ICMT) (Figure 4) (reviewed in Prior and Hancock 2001, and in Hancock 2003). These post-translational modifications also will determinate the trafficking route of these proteins towards the membrane. For the K-RAS4B protein, these modifications are sufficient for the membrane binding, so the K-RAS4B undergoes a Golgi-independent route, whereas the other RAS proteins require a palmitoylation step, which is performed in the Golgi complex - Golgi-dependent route (Figure 4) (reviewed in Prior and Hancock 2001, in Hancock 2003, and in Karnoub and Weinberg 2008).

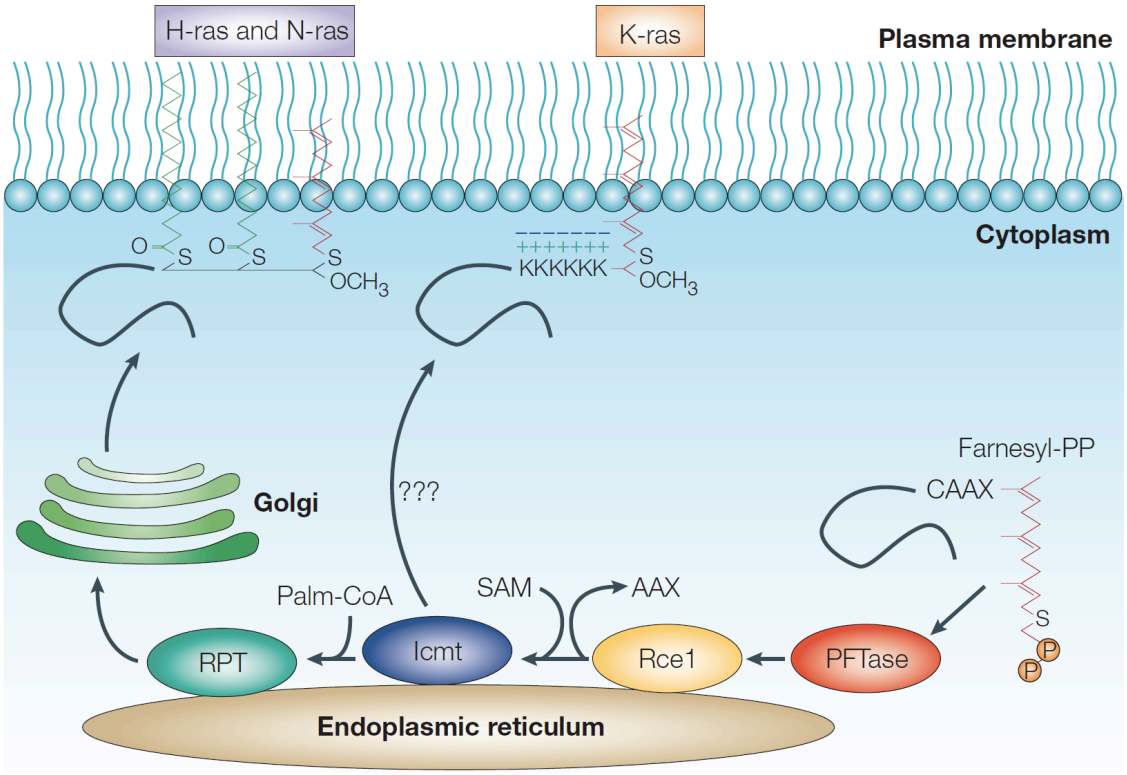


Figure 4 - Post-translation modifications of RAS proteins (adapted from Hancock 2003). The p21RAS protein undergoes post-translational modifications (prenysilation of the C-terminal cysteine, followed by cleavage of the AAX motif and methylation of the farnesylated cysteine), in order to be targeted to the membranes.

The p21RAS is located mostly at the plasma membrane, but it has also been

shown that p21RAS can be present in the Golgi complex, and several of their effectors where found there, suggesting that the Golgi complex could be a site of active RAS signalling. N-RAS and K-RAS4B were also identified in the mitochondria, and several studies suggested that these proteins have a crucial role in the mitochondrial morphology (reviewed in Prior and Hancock 2001, in Hancock 2003, and in Omerovic *et al*., 2007). p19H-RAS was found in the cytoplasm membrane and nucleus, due to the lack of HVR in this protein (Guil *et al*., 2003a; Kim *et al*., 2008).

Studies using membrane destabilizing drugs showed that p21RAS proteins have a differential distribution in the plasma membrane. These studies show that different p21RAS proteins would interact with different pools of effectors, even though these proteins are able to interact with all the same effectors, but the differential distribution in the plasma membrane could accounts for this difference. This occurs because the effectors and regulators of RAS are differentially distributed, allowing preferential coupling to specific signalling pathways in each subcellular location (reviewed in Omerovic and Prior 2009).

1.3.3 – The RAS proteins functions

As mentioned in chapter 1.3, p21RAS are GTP-binding proteins, with an intrinsic GTPase activity, that function as molecular switches, being the active form bound to GTP and the inactive form bound to GDP (reviewed in Lim *et al*., 1996, and in Takai *et al*., 2001) (Figure 4). These characteristics combined with the capacity and the ability to bind to target proteins, offer a mechanism of downstream transmission of external mitogenic signals, as it occurs in the MAPK pathway (reviewed in Malumbres and Barbacid 2003, and in Karnoub and Weinberg 2008). The different signalling cascade where p21RAS (H-RAS, N-RAS and K-RAS4B) proteins are involved may differ according the different RAS genes that are expressing them, but generally they lead to the induction of cell cycle regulation, cellular proliferation, differentiation, growth arrest

(Figure 5) (reviewed in Karnoub and Weinberg 2008). The p21RAS was also been shown to interact with RASSF, a pro-apototic protein (Khokhlatchev *et al.*, 2002), although the mechanism associated with this signalling pathway remains unclear (Figure 5) (reviewed in Karnoub and Weinberg 2008).

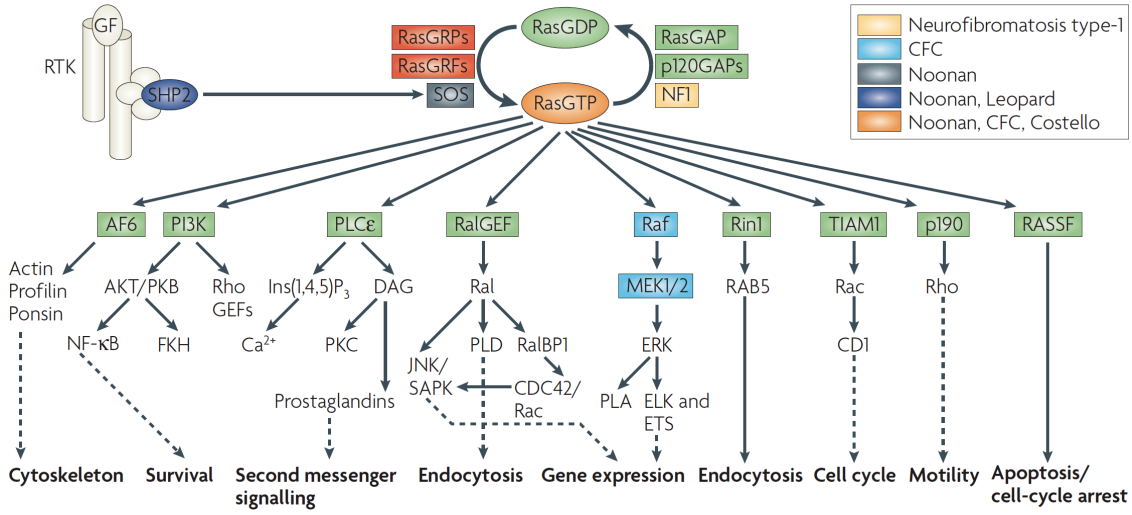


Figure 5 - RAS signalling network and outcomes (adapted from Karnoub and Weinberg 2008). The p21RAS proteins are effects of several signalling pathways, that mostly lead to gene expression, cell cycle regulation, proliferation and motility, although this protein also interacts with proteins involved in the apoptosis (RASSF), although this pathways remains unclear. These proteins, due to their importance in the cell cycle progression and regulation are associated with several diseases.

1.3.4 – The role of RAS proteins in cancer

The RAS genes are major participants in the development and progression of different types of human cancers, such as gastrointestinal cancers, lung cancer, breast cancer, bladder cancer, thyroid cancer, kidney cancer, pancreatic cancer, among others (Table II) (reviewed in Nishimura and Sekiya 1987, in Bos 1989, Anderson *et*

al., 1992, and in Karnoub and Weinberg 2008). Several reports have shown the importance that the RAS genes have in tumour formation (Ise *et al*., 2000), maintenance (Chin *et al*., 1999), and stimulation of angiogenesis (Arbiser *et al*., 1997).

1.3.4.1- RAS oncogenic activation

Different studies demonstrated that point mutations in the RAS genes appear in a large number of tumours (reviewed in Bos 1989) and it have also been detected in pre-malignant lesions, suggesting that the activation of these genes may be an early event in the tumourigenic process (reviewed in Brandt-Rauf 1991). Several point mutations in the RAS genes have been associated with the increase of transformation potential in cells, being these mutations in the codons 12, 13 and 61 (Fujita *et al*., 1985; Visvanathan *et al*., 1988; Vogelstein *et al*., 1988; Lemoine *et al*., 1989; Shi *et al*., 1991; Nikiforova *et al*., 2003; Soares *et al*., 2003). The mutations in these specific locations will have an important role in the p21RAS activity, since they will decrease its intrinsic GTPase activity, maintaining the RAS in the active form for a longer period of time (reviewed in Bos 1989, and in Malumbres and Barbacid 2003). These mutations are designated as dominant positive mutations because of their potential to lock the RAS protein in the active form, continuously interacting with their effectors, leading to a continuous activation of the signalling pathways due to prevention of the normal deactivation of these proteins (Krengel *et al*., 1990; Wittinghofer *et al*., 1991; Scheffzek *et al*., 1997).

Table II - Incidence of RAS mutations in human cancers, according to the Catalogue of Somatic Mutations in Cancer (Cosmic) of the Wellcome Trust Sanger Institute, Cambridge, UK (adapted from Karnoub and Weinberg 2008). ND- not determined.

Tissue	H-Ras	K-Ras	N-Ras
Adrenal gland	1%	0%	5%
Biliary tract	0%	32%	1%
Bone	2%	1%	0%
Breast	1%	5%	1%
Central nervous system	0%	1%	2%
Cervix	9%	8%	1%
Endometrium	1%	14%	0%
Eye	0%	4%	1%
Gastrointestinal tract (site indeterminate)	0%	19%	0%
Haematopoietic and lymphoid tissue	0%	5%	12%
Kidney	0%	1%	0%
Large intestine	0%	32%	3%
Liver	0%	7%	4%
Lung	1%	17%	1%
Meninges	0%	0%	0%
Oesophagus	1%	4%	0%
Ovary	0%	15%	4%
Pancreas	0%	60%	2%
Parathyroid	0%	0%	0%
Peritoneum	0%	6%	ND
Pituitary	2%	0%	0%
Placenta	0%	0%	0%
Pleura	0%	0%	0%
Prostate	6%	8%	1%
Salivary gland	16%	4%	0%
Skin	5%	2%	19%
Small intestine	0%	20%	25%
Stomach	4%	6%	2%
Testis	0%	5%	4%
Thymus	0%	15%	0%
Thyroid	4%	3%	7%
Upper aerodigestive tract	9%	4%	3%
Urinary tract	12%	4%	3%

Data derived from the Catalogue of Somatic Mutations in Cancer (COSMIC) of the Wellcome Trust Sanger Institute, Cambridge, UK. ND, not determined.

1.3.4.2 – Wild-type protein over-expression

The effect of mutations in the RAS genes is well studied; nonetheless the role of wild-type RAS in the cell transformation is still unclear. The members of this family exist in low levels in the cells, being the highest levels of RAS protein found in proliferating cells (reviewed in Denhardt 1996). Therefore, when inducing a moderate increase of the RAS levels, the effects can be observed by the rapid growth of the cells. The over-expression of the normal RAS gene, when transfected to cell lines, has shown to increase the tumourigenicity of the cells and even induce the formation of tumours (Table III) (Pulciani *et al*., 1985). On the other hand, other studies have reported that the wild-type RAS can act as a negative regulator of the mutated form. When cells with T24 mutant H-RAS or the mutant N-RAS were transfected with the normal HRAS gene, it occurs a reversion of the transforming and tumourigenic phenotype of the cells (Spandidos and Wilkie 1988; reviewed in Spandidos *et al*., 2002). The mechanism involved in this outcome is not yet known, but several theories have been raised, like that the wild-type RAS competes with the oncogenic form for the effectors, at membrane level; the normal RAS gene can lead to activation of p53 or p16 or other protein that promotes cell cycle arrest; or the normal RAS gene can promote differentiation (reviewed in Spandidos *et al*., 2002; Singh *et al*., 2005).

Table III - Transforming phenotype and tumour induction of cells transfected with a high number of normal RAS gene copies (adapted from Pulciani *et al.*, 1985). The cells transfected with a high number of copies of the normal RAS gene present higher tumour induction.

Cell line	Transfected H-*ras* gene	No. of gene copies	Tumorigenicity			Level of expression of:	
			Phenotype	Growth in soft agar	Tumor induction[a]	H-*ras* RNA[b]	p21 *ras*[c]
134-51	spontaneously activated oncogene[d]	1	Transformed	+	5/5	1×[e]	1×[e]
109-31	T24 oncogene	1	Transformed	+	5/5	10×	20×
115-11	Proto-oncogene	30	Transformed	+	3/10	20×	20×
115-14	Proto-oncogene	>50	Transformed	+	10/10	80×	50×
115-16	Proto-oncogene	>50	Transformed	+	10/10	80×	50×
136-41	Proto-oncogene	>50	Transformed	+	10/10	ND[f]	ND
NIH 3T3			Normal	−	0/10	0.3×	0.4×

[a] NFS/N weanlings were subcutaneously injected with 10^6 cells. Only nonregressing tumors were scored.
[b] See Fig. 3 for a representative experiment.
[c] Based on immunoprecipitation analysis of ^{35}S-labeled proteins. See Fig. 4 for a representative experiment.
[d] See reference 23.
[e] 134-51 cells are taken as reference because they contain the lowest level of oncogene expression of all NIH 3T3 transformants tested in our laboratory, thus representing the minimal amount of H-*ras-1* oncogene expression necessary to transform NIH 3T3 cells.
[f] ND, Not determined.

1.3.5 – The clinical relevance of H-RAS gene

Germ-line mutations in H-RAS gene cause Costello syndrome, a syndrome of mental and growth retardation (Johnson *et al.*, 1998). The inherited mutations associated with this syndrome are mutations in codon 12 (Gly12Ser) and in codon 13 (Gly13Cys), that lead to the production of p21RAS proteins permanently active (Aoki *et al.*, 2005; Estep *et al.*, 2006). The mechanism that leads to this disease is yet unclear. These patients, during childhood and adolescence, are more prone to develop benign tumours, such as papillomata, and cancers, inclunding rhabdomyosarcoma, ganglioneuroblastoma and bladder cancer (reviewed in Hennekam 2003, and in Gripp 2005).

It is also known that somatic mutations in the codons 12, 13 and 61 of the H-RAS gene can lead to the oncogenic activation of p21RAS, and that these mutations are associated to bladder (Fujita *et al.*, 1984), kidney (Visvanathan *et al.*, 1988) and thyroid cancer (Lemoine *et al.*, 1989; Bouras *et al.*, 1995).

1.3.5.1 – Alternative splicing of H-RAS gene

Cohen was the first author to describe the alternative splicing process of H-RAS in 1989 (Cohen *et al*., 1989). The H-RAS mRNAs can be alternatively processed in two different proteins, due to the inclusion or exclusion of the alternative exon IDX (Figure 6), an exon located in the intron D, between exon 3 and exon 4A of the H-RAS pre-mRNA (Codony *et al*., 2001). The exon IDX has a stop codon, which leads to a stop on translation, originating a protein with 19KDa - the p19H-RAS protein, smaller than p21RAS due to the lack of the exon E4A (Figure 6) (Codony *et al*., 2001).

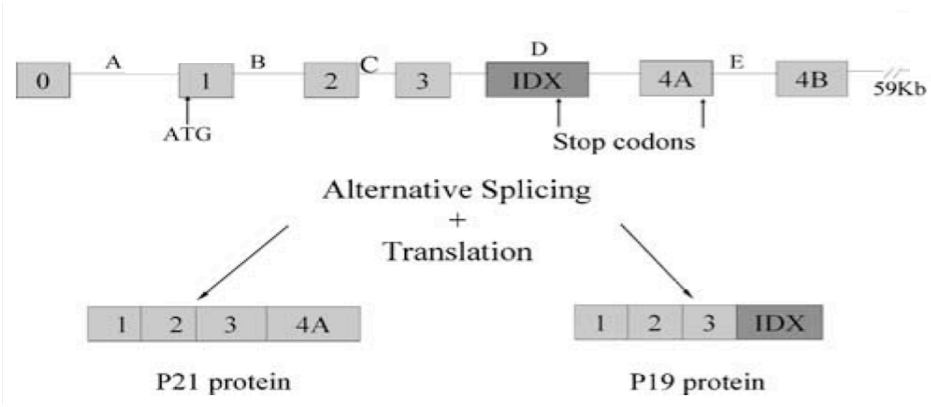


Figure 6 - Alternative splicing in the H-RAS (Adapted from Castro *et al*., 2006).

It is not yet clear how this process occurs, even though several studies have been trying to clarify this process and several theories have been proposed. Guil *et al* (Guil *et al*., 2003b), proposed the existence of a sequence downstream of the IDX exon – rasISS1- that can negatively regulate the inclusion of IDX exon. They also showed that this process could be negatively affected by p68 helicase and hnRNP A1 protein; and positively affected by two SR proteins, SC35 and SRp40 (Guil *et al*., 2003b). Later, it was found that p68 helicase could interfere in several aspects of the alternative splicing process, like by affecting the structure of the stem-loop IDX-rasISS1,

increasing the levels of SR35 protein available and disrupting the binding of hnRNP H, a protein that appears to be necessary for IDX inclusion (Camats *et al*., 2008). It was also demonstrated that FUS/TLS protein may stimulate IDX inclusion (Figure 7) (Camats *et al*., 2008).

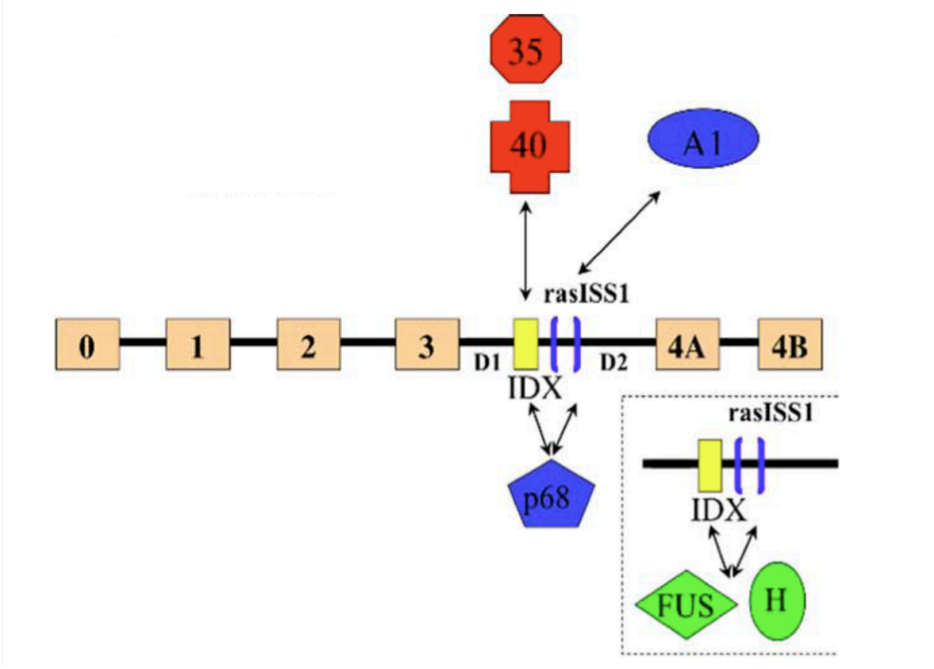


Figure 7 - Regulation of the alternative splicing process in H-RAS gene (adapted from Camats *et al*., 2008)**.** The p68 helicase and hnRNP A1 protein (in red) negatively regulate the inclusion of exon IDX and the SC35 and SRp40 (in green) positively regulate the inclusion of IDX. The FUS/TLS although it doesn't bind directly to the rasISS1 sequence, it appears to stimulate IDX inclusion; and hnRNP H binds to rasISS1 and it is necessary for IDX inclusion.

1.3.5.2 – p19H-RAS proteins

Cohen's described the p19H-RAS in 1989 (Cohen *et al*., 1989) but only recently this protein has started to be studied. p19H-RAS mRNA is stable and p19H-RAS protein is active and present in many mammalian species, in several cell lines and

human tissues (Guil *et al*., 2003a). The p19H-RAS is distributed in the nucleus and cytoplasm, contrarily to other members of the RAS family, since the C-terminal conserved domain (located at exon 4), that targets the proteins to the plasma membrane, is no translated due to the stop codon located at exon IDX (Guil *et al*., 2003a; Kim *et al*., 2008).

It was also shown that p19H-RAS doesn't interact with two known p21RAS effectors - RAF1 and RIN1, or with p21RAS, although it is able to interact with itself (Guil *et al*., 2003a). The lack of interaction with the known p21RAS effectors is explained by the fact that p19H-RAS lacks the E4A domain, where the two GTP-binding sites are located, therefore p19H-RAS present a diminished GTP-binding activity (Guil *et al*., 2003a). p19H-RAS interacts with RACK1, a scaffolding protein that promotes multiprotein complexes in different signalling pathways (Guil *et al*., 2003a). The meaning of this interaction needs further clarification.

It was also demonstrated that p19H-RAS can interact with p73β in the nucleus (Jeong *et al*., 2006), leading to apoptosis, by the amplification of p73β-induced apoptotic signalling responses - BAX mitochondrial translocation, cytochrome c release, production of reactive oxygen species and losing of mitochondrial transmembrane potential (Kim *et al*., 2008); p73β also regulates telomerase activity (Camats *et al*., 2009). The p73 is a p53-related protein, which has an important role in cellular damage response, and because it shares a high degree of sequence homology and many structural features with p53, allows the p73 to bind consensus p53 binding sites and it activates the expression of repair genes. The p73 protein is also an inducer of the cell cycle arrest and apoptosis, although the mechanism that leads to the cell death is yet unclear (reviewed in Kaelin 1999). The p19H-RAS was shown to enhance the transcriptional activity of p73β by blocking MDM2-mediated p73β repression (Jeong *et al*., 2006). MDM-2 (mouse double minutes 2) is an oncoprotein, which is known to induce p53 degradation through ubiquitin-mediated proteolysis and it can also bind to p73, leading to an inhibition of the p73 transactivating activity, even though it doesn't

induce p73 degradation (reviewed in Daujat *et al*., 2001). The p19H-RAS has also the ability to interact with p53 and p73, and can also be activated by agents used in chemotherapy, such as taxol, which upregulates p73 mRNA and stability, leading to the increase of p73 levels and apoptosis (Kim *et al*., 2008).

A recent study (Jang *et al*., 2009) showed that the neuron-specific enolase (NSE) also can bind to the C-terminal region of the p19H-RAS, inhibiting the NSE function and leading to the blockage of the glycolytic pathway and to the decrease of the proliferation enhanced by the NSE activity (Jang *et al*., 2009). The NSE, also known as enolase 2, is a glycolytic enzyme that catalyzes the inter-conversion of 2-phospho-D-glycerate (2PG) in phosphoenolpyruvato (PEP) (reviewed in Kim and Dang 2005), that can be found in the brain and lung cancer tissues, being the high levels of NSE associated with tumour grade and proliferative activity in lung cancer, leading to a worse prognosis (Ferrigno *et al*., 1994). The p19RAS was also shown to interact and block AChE-R (enhancing enzymatic activity of enolase), an enzyme that can stimulate the activity of NSE (Jang *et al*., 2009).

The p19H-RAS is also involved in the G1/S phase delay, through the hypophosphorylation of AKT and upregulation of FOXO1, leading the cells to a reversible quiescence state and preventing the entry in apoptosis (Camats *et al*., 2009).

All of the data we have on p19RAS show that this protein behaves more like a tumour suppressor, further studies are needed to clarify this issue.

1.3.5.3 – H-RAS 81T-C polymorphism and tumourigenesis

The H-RAS 81T-C polymorphism, first described by Taparowsky *et al* (1982), is a polymorphism, found in high frequency, which occurs in the first exon of the H-RAS gene (Figure 8).

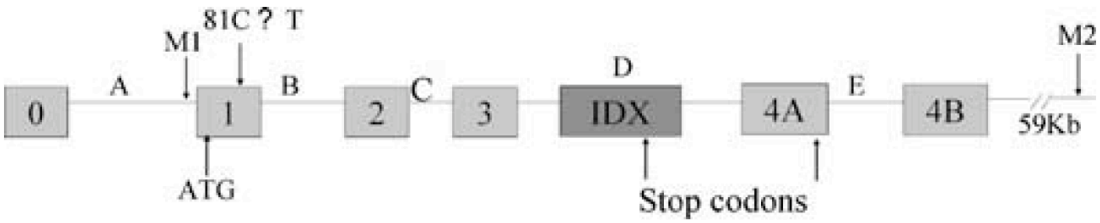


Figure 8 - Scheme of H-RAS gene, showing the location of 81T-C polymorphism (Castro *et al*., 2006).

Several observations showed that the T allele has an higher frequency, in comparison with the C allele, and the frequency of the genotypes is quite different between the populations (Table IV).

Table IV – Frequency of the presence of the H-RAS 81T-C in different populations.

Type of tumours	Frequency of the presence of the C allele		Country	Study
	Patient Group	Control Group		
Bladder tumours	51.6%	58.3%	Germany	(Johne *et al*., 2003)
Thyroid tumours	62.4%	51.0%	Portugal	Castro *et al*., 2006)
Oral cancer	46.6%	35.2%	India	(Sathyan *et al.*, 2006)
Gastrointestinal cancer	31.1%	20.8%	China	(Zhang *et al.*, 2008)
Colon cancer	39.5%	57.5%	Croatia	(Catela Ivkovic *et al.*, 2009)

This polymorphism (the presence of the C allele) has been associated with an increase risk of several types of cancer, such as: development and progression of bladder cancer (Johne *et al*., 2003), oral cancer (Sathyan *et al*., 2006; Murugan *et al*.,

2009), gastric cancer (Zhang *et al*., 2008) and colon cancer (Catela Ivkovic *et al*., 2009). The H-RAS 81T-C polymorphism has also been associated with alterations in the ploidy status in follicular thyroid tumours and in nodular goitres, towards an increase in the aneuploidization of these tumours (Castro *et al*., 2006). These findings were also show in gastric tumours and bladder tumours, associating the H-RAS 81T-C polymorphism with aneuploidization (Castro *et al*., data unpublished).

The H-RAS 81T-C polymorphism does not lead to alterations in the p21RAS structure, since it is located in a wobble base position (Johne *et al*., 2003; Sathyan *et al*., 2006). However, it was observed that H-RAS 81T-C polymorphism is associated with the alternative splicing process of the H-RAS gene and, therefore, altering the ratio of p21RAS/p19RAS, towards an increase of p21RAS (Castro *et al*., 2006). The increase of p21RAS expression can be responsible for the observed aneuploidy induction (Castro *et al*., 2006), since the p21RAS over-expression is associated with an increase in the tumourigenicity (Pulciani *et al*., 1985) and the RAS oncogenes have been shown to induce chromosome missegregation (Hagag *et al*., 1990; Saavedra *et al*., 1999) and generation of abnormal chromosome content in cancer cell lines (Denko *et al*., 1994; Saavedra *et al*., 1999), through the MAPK pathway (Saavedra *et al*., 1999). The genetic mechanism of the H-RAS polymorphism is yet unclear, although some hypotheses have been proposed. H-RAS minisatellite, located about 1kb from exon 4, may influence H-RAS transcription (Trepicchio and Krontiris 1992). Other hypothesis is that H-RAS 81T-C polymorphism could serve as a marker for a polymorphism in intron D2, responsible for the regulation of IDX exclusion (Castro *et al*., 2006), but after sequencing the whole gene the latter hypothesis was disregarded (Castro *et al*., unpublished results).

1.4 – Aims

The overall goal of this work is to better understand how the H-RAS 81T-C

polymorphism affects cellular behaviour.

1.4.1 – To evaluate the effects of the H-RAS 81T-C polymorphism in the splicing of the H-RAS gene

In order to evaluate the effect of the 81T-C polymorphism in the splicing of the H-RAS gene we studied the expression of the p21H-RAS and p19H-RAS and the p21H-RAS/p19H-RAS ratio expressed by HEK293 cells transfected with an H-RAS mini-gene, carrying the T or the C allele. We have assessed this through real-time RT-PCR, using validated probes for p21H-RAS and p19H-RAS mRNA. We could not access the protein levels of the two isoforms since there are no antibodies targeting the p19H-RAS.

1.4.2 – To evaluate the effects of the H-RAS 81T-C polymorphism in the chromosomal content and chromosomal instability of the cells

In order to study the effect of the H-RAS 81T-C polymorphism in the chromosomal content and chromosomal stability we treated the HEK293 cells transfected with the both allelic forms of the H-RAS mini-gene with an hypotonic solution, to obtain chromosomal spreads of the mitotic cells and count the chromosomes. We also studied the centrosome amplification in these cells, by immunofluorescence, using a pericentrin antibody.

1.4.3 – To evaluate some tumourigenic parameters in the cells over-expressing each one of the H-RAS 81 alleles

In order to study some classic tumourigenic parameters in the cells transfected with both forms of the H-RAS mini-gene we evaluated the following features: cell

growth, proliferation, cell death and migration. For evaluation of the cell growth we performed the sulforhodamine B (SRB) assay. The results of this evaluation have been tested together with proliferation, assessed by the BrDU proliferation assay; and the cell death, assessed by the TUNEL assay. The evaluation of the cell migration was performed using the wound-healing assay.

Chapter 2 Materials and Methods

2.1 – Materials

The cells were cultured in Dulbecco's Modified Eagle Medium (DMEM), fetal bovine serum (FBS), TrypLE™ Express, Geneticin G-418, Penicillin Streptomycin (Pen Strep), Ampotericin B (Fungizone) were purchased from GIBCO, as a part of Invitrogen Life Technologies (California, USA). DNAse I kit, RevertAid™ M-MuLV Reverse Transcriptase, Random Hexamer Primer, RiboLock™ RNase Inhibitor were acquired from Fermentas. The dNTPs mix was purchased from Bioron. The Taqman Gene Expression Assays for the p21H-RAS (Hs00610483_m1), p19H-RAS (Hs00978053_g1) and GAPDH (433764T) were acquired from Applied Biosystems (Foster City, CA, USA). Albumin Fraction V (from bovine serum) was acquired from AppliChem (Darmstadt, Germany). The DC Protein Assay was acquired from Bio-Rad (Hercules, CA, USA). The developer and the fixer were purchased from Kodak (Rochester, NY, USA). Vectashield mounting medium containing DAPI was acquired from Vectorlabs (Burlingame, CA, USA). The In Situ Cell Death Detection Kit, Fluorescein and the TUNEL dilution buffer were acquired from Roche (Manheim, Germany). The X-ray film was purchased from GE Healthcare Life Sciences (Uppsala, Sweden). All other reagents of high purity were purchased from Sigma-Aldrich (Missouri, USA).

The Luminol and the P-Cumaric Acid were made in DMSO solutions; all the other chemicals were maintained in aqueous solutions.

2.2 – Methods

2.2.1 – Plasmid construction

The human embryonic kidney cell line, HEK293, was transfected in order to generate stable cells using the Tol2 transposon method. This method uses a binary co-

transfection assay with the simultaneous transfection of two vectors: a Transposon Donor Plasmid that contains a drug selection marker (Neo), a strong promoter (CMV1), the H-RAS full-length gene carrying the T or the C allele all flanked by the Tol2 transposion arms (Figure 8). It also contains two genes conferring resistance to the ampicillin and kanamycin antibiotics and an origin of replication. The other vector used by this system is a Helper Plasmid that holds the transposase cDNA under the control of a CMV promoter. The transposase will recognize and cut the *Tol2* arms. The HEK293 cells were also transfected with a mock vector lacking the H-RAS gene.

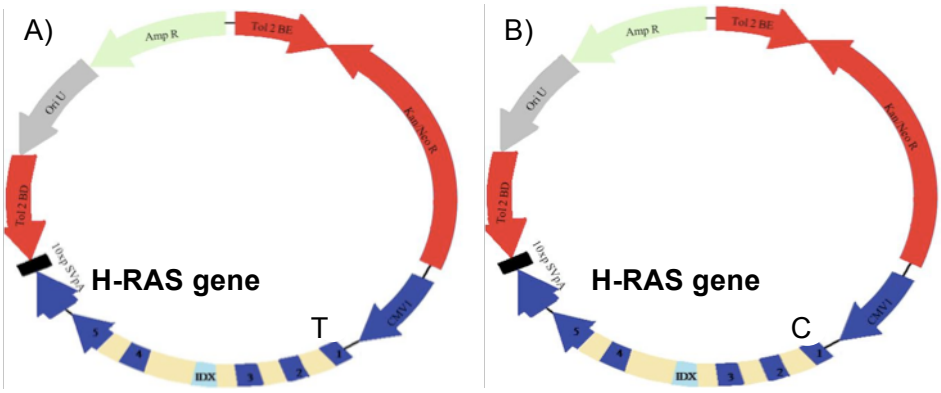


Figure 9 – Scheme of the vectors containing the H-RAS mini-gene. A) H-RAS mini-gene carrying the T allele in position 81. B) H-RAS mini-gene carrying the C allele in position 81.

The selection of the stable cells was done using 500 µg/l of G-418 added to the DMEM complete medium and the posterior maintenance of the stable cells was done using 200 µg/l of G-418.

The vectors were constructed and transfected into the HEK293 cells by Luís Costa and Isabel Pereira-Castro.

2.2.2 – Cell culture

The transfected HEK293 cells were used in all experiments and it were maintained in standard conditions (37ºC and 5% CO2). The transfected HEK293 cells were cultured Dulbecco's Modified Eagle Medium (DMEM), supplemented with Fetal Bovine Serum (10% v/v), Pen Strep (1% v/v), Fungizone (0,5% v/v) and G-418.

2.2.3 – Real-time quantitative PCR

The total RNA was extracted from transfected HEK293 cells, and it was treated with DNase I kit (Fermentas), according with the manufacture instructions. Briefly, it was add 1ug of RNA, 1x reaction buffer with $MgCl_2$ and DNase I 1μL for 1μg RNA, to a 10μL volume reaction, incubated for 30 min at 37ºC, and afterwards incubated with 25mM EDTA for 10 min at 65ºC. The reactions were performed on a Bio-Rad MyCycler™ Thermal Cycler. The cDNA synthesis was performed with 1μg of the treated RNA, using 100ρmol random hexamer primer (Fermentas), 200u RevertAid™ M-MuLV Reverse Transcriptase (Fermentas), 5x reaction buffer (Fermentas), 20u RiboLock™ RNase Inhibitor (Fermentas) and 1mM dNTPs mix (Bioron), to a final volume of 20μL. The thermal cycling conditions were 10 min at 25ºC, followed by 60 min at 42ºC and finally 10 min at 70ºC. The reactions were performed on a Bio-Rad MyCycler™ Thermal Cycler.

The Real-time assay was performed on an Applied Biosystems 7500 Fast Real-time PCR System. Validated Taqman Gene Expression Assays (Applied Biosystems) were mixed with Taqman mix (Applied Biosystems) and 1μg of RNA, and incubated following the thermal cycling conditions: 10 min at 95°C, followed by 40 cycles of 15s at 95°C and 1min at 60°C.

2.2.4 – Total extracts preparation and quantification

The transfected HEK293 cells were washed with ice-cold phosphate buffered saline – PBS (137mM NaCl, 2,7mM KCl, 10mM Na_2HPO_4, 1,8mM KH_2PO_4, pH7.4) and incubated with lysis buffer. The lysates were then centrifuged at 1400rpm during 10 minutes at 4ºC, the supernatants were recovered and stored at -20ºC. The protein lysates were quantified with the DC Protein Assay (Bio-Rad), using albumin (BSA) dilutions to construct the standard curve. The lysates were diluted in water and 4x concentrated denaturing buffer and denaturated at 98ºC for 5 min.

2.2.5 – Antibodies

The rabbit p44/42 MAPK (ERK 1/2) antibody and rabbit phospho-p44/42 MAPK (phospho-ERK 1/2) antibody were acquired from Cell Signaling Technology (Danvers, MA, USA); the polyclonal goat β-actin antibody and the secondary antibodies were purchased from Santa Cruz Biotechnology, Inc (Santa Cruz, CA, USA). The monoclonal rabbit GTPase H-RAS antibody and the polyclonal rabbit to pericentrin antibody were purchased to Abcam (Cambridge, UK). The monoclonal mouse anti-bromodeoxyuridine (anti-BrDU) clone Bu20a antibody, the polyclonal swine anti-rabbit Ig-FITC antibody, the polyclonal rabbit anti-mouse Ig-FITC antibody were acquired from Dako (Glostrup, Denmark).

2.2.5.1 – Western blot

The denatured extracts were resolved on a SDS-PAGE 12% poliacrylamide gel, during approximately 2h at 100V. The electrotransfection onto a nitrocelulose membrane was performed during 2h at 100V. 50μg of protein were loaded in the gels. The membranes were blocked using phosphate buffered saline – PBS with 0.04%

Tween20 (PBS-T) and 5% non-fat dry milk for 30 min. Then the membranes were incubated for 1h at room temperature or overnight at 4ºC, with the primary antibody in PBS-T and 5% milk (for the β-actin, H-RAS and ERK 1/2) or with the primary antibody in PBS-T and 5% BSA (for the phospho-ERK1/2). After, the membranes were washed and incubated with alkaline phosphatase conjugated IgG secondary antibody (in accord with the primary antibody host specificity) in PBS-T with 5% milk for 45 min at room temperature. Then, the membranes were washed with PBS-T and were developed using Enhanced Chemiluminescence (ECL) reagent hand-maid (the solution 1 is constituted by 2.5mM Luminol, 400μM P-Cumaric acid, 100mM Tri-HCL pH8.6; the solution 2 is constituted by 0.15% H_2O_2, 100Mm Tris-HCl pH8.6). The results appear after expose the X-ray film (GE Healthcare Life Sciences) to the chemiluminescent membranes and emerging it in developer and fixer (KODAK). For subsequent probing of the membranes, the membranes were stripped of the antibodies using a stripping solution with for 30 min, at room temperature, washed several times with PBS-T, blocked with PBS-T with 5% milk and reprobed for the primary antibodies.

2.2.5.2 – Immunofluorescence for pericentrin

The cells were plated in coverslides and were grown at 37ºC and 5% CO_2 until 80-90% of the confluence was reached. After the protocol recommended by the manufactured was followed. Briefly, the cells were fixated using 3.5% paraformaldhyde with 0.002M NaOH, 10 mL of 10xPBS, 10mM PIPES; and washed with KB (0.01 Tris pH 7.5, 0.15M NaCl, 0.1% BSA) with 0.2% Triton X-100, and with KB. The cells were then incubated with 1 μg/ml rabbit polyclonal to pericentrin antibody (Abcam), for 30 min at 37ºC. Afterwards, the cells were washed with KB and incubated with the corresponding secondary antibody – polyclonal swine anti-rabbit Ig-FITC (Dako) – and washed with KB. The antibodies were diluted in KB. Counterstaining was performed with Vectashield mounting medium containing DAPI (Vectorlabs, Burlingame, CA). The

slides were then observed at the fluorescence microscopy Zeiss Axio Imager 2.0 microscope (Zeiss) and the imaging was study using the Zeiss AxioVision LE software (Zeiss).

2.2.6 – Hypotonic treatment

The cultured cells were ressuspended in the culture medium, centrifuged and washed in PBS. The cells were then incubated in 0.075M KCl solution for 20-30 min, and centrifuged. Then, the cells were fixed in a fixative solution (methanol and glacial acetic acid, in a 4:1 ratio), for 30min at room temperature, and centrifuged. The fixative step was repeated 2-4 times. The cells were then stored in fixative solution, at -20ºC, and later, the slides were mounted by dropping one drop of the stored cells in a heated slide (37ºC), let it dry and stained the cells with Vectashield mounting medium containing DAPI (Vectorlabs, Burlingame, CA). The cells were observed at the fluorescence microscopy Zeiss Axio Imager 2.0 microscope (Zeiss) and the imaging was study using the Zeiss AxioVision LE software (Zeiss).

2.2.7 – Sulforhodamine B assay

The cells were seeded in a 96-well plate and cultured during 3 days. After that time, the cells were fixated using 50% trichloroacetic acid for 1h at 4ºC. The fixed cells were washed with tap water 4 times and air dried. Then, the cells were incubated with 0.1% Sulforhodamine B diluted in 1% glacial acetic acid, during 30 min at room temperature and were washed 4 times with tap water and were air dry. The stained cells were then solubilized with Tris-base buffer pH 10.5 and incubated during 30 min. Afterwards the absorbance was measured at 560 nm, on a Bio-Rad Model 680 Microplate Reader.

2.2.8 – BrDU proliferation assay

The cells were incubated with BrDU (10μM; Sigma-Aldrich) for 1h and then the cells were washed with ice cold PBS, fixated using 4% paraformaldehyde (Sigma) for 15 min, washed in PBS and stored in PBS at 4ºC. Afterwards, the cells were treated with 2M HCl for 20 min at room temperature and washed with PBS. The cells were then blocked in PBS with 0.5% Tween 20 and 0.05% BSA (PBS-T-B) for 5 min at room temperature and incubated with the anti-BrDU (Dako) for 1h at room temperature. Afterwards the cells were washed 3 times with PBS-T-B and incubated with the secondary antibody targeting specifically the host of the primary antibody for 30 min at room temperature.

2.2.9 – TUNEL assay

The cultured cells were fixed in 4% paraformaldhyde, during 15min and stored at 4ºC. Afterwards, the cells were treated with a permeabilization solution (0.1% Triton X-100 and 0.1% sodium acetate) and incubated in a mix containing the Enzyme solution and the Label solution, from the In Situ Cell Death Detection Kit, Fluorescein (Roche), diluted in the TUNEL dilution buffer (Roche) - for 1h at 37ºC. The slides were then prepared, by counterstaining the cells with Vectashield mounting medium containing DAPI (Vectorlabs), and examined in the Zeiss Imager Z1 and the photographed by Zeiss AxioCam Mrm.

2.2.10 – Wound healing assay

The cells were plated until reach confluence. After, a scratch was performed in the cell monolayer using a pipet tip and the cell migration was monitored by time-lapse,

every 5 min, for 12h, in the Leica DMIRE2 inverted microscope and filmed by the Leica DFC350 FX camera. The films were analysed using the Zeiss LSM Image Browser.

2.2.11 – Statistical analysis

The data was analyzed using Microsoft Office Excel (Microsoft). The statistic analysis was assessed using the T-test. P-values >0.05 were considered significant.

Chapter 3 Results

3.1 – The influence of the H-RAS 81T-C polymorphism in the H-RAS alternative splicing

We decided to stable transfect the HEK293 cells with two version of the H-RAS mini-gene (the whole gene, from exon 1 to exon 4) (Figure 10A): the first version has the allele T in the position 81 and the other has the allele C in the position 81. We also transfected the HEK293 cells with the empty vector (Figure 10B) that will serve the control cell-line (mock cells). The decision to use the HEK293 cells were mainly two: it is a non-tumourigenic cell-line easy to transfect and it is homozygous for the T allele of the H-RAS 81 T-C polymorphism. This strategy will eliminate the cellular background, since we are working with the same cell-line that only differ in the H-RAS at position 81. After the transfection of the HEK293 cells and the selection of stable clones with the G-418 antibiotic, we sequenced the mRNA of the H-RAS gene, in order to confirm if the H-RAS mini-gene was being expressed. Our results confirm that the HEK293 cells transfected with the H-RAS mini-gene carrying the T and the C alleles were indeed expressing the T allele (Figure 10D) and the C allele (Figure 10E), respectively. In the HEK293 transfected with the H-RAS C allele we were not able to detect the T allele, either in the DNA or in the cDNA, showing that the transfection efficiency was very high, therefore beyond the capacity of the sequencing detection. We also observe that the HEK293 cells transfected with the empty vector were expressing the normal H-RAS phenotype of this cell line, the T allele (Figure 10C).

After stable transfecting HEK293 cells with H-RAS mini-gene carrying the T allele, with the H-RAS mini-gene carrying the C allele and with the empty vector (Mock), we quantify the mRNA levels of the H-RAS gene in the three derived cell lines. This allowed us to evaluate if the mini-gene transfected into the HEK293 cells was producing a high amount of H-RAS mRNA.

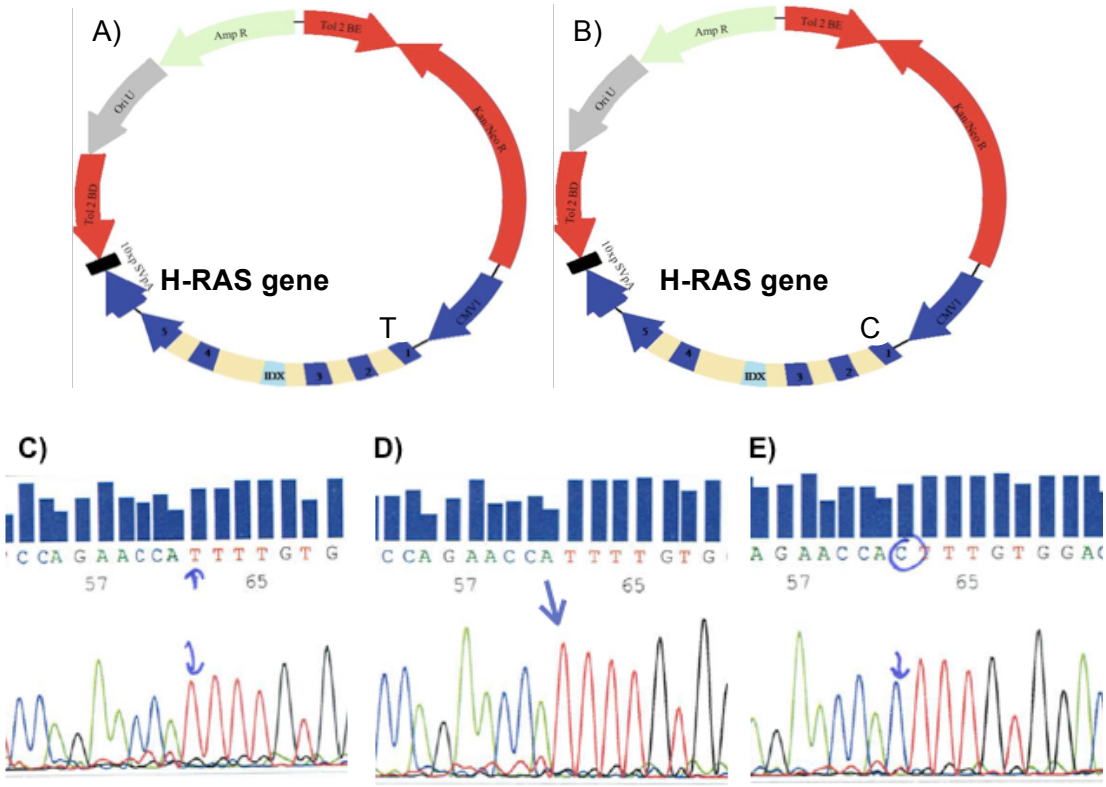


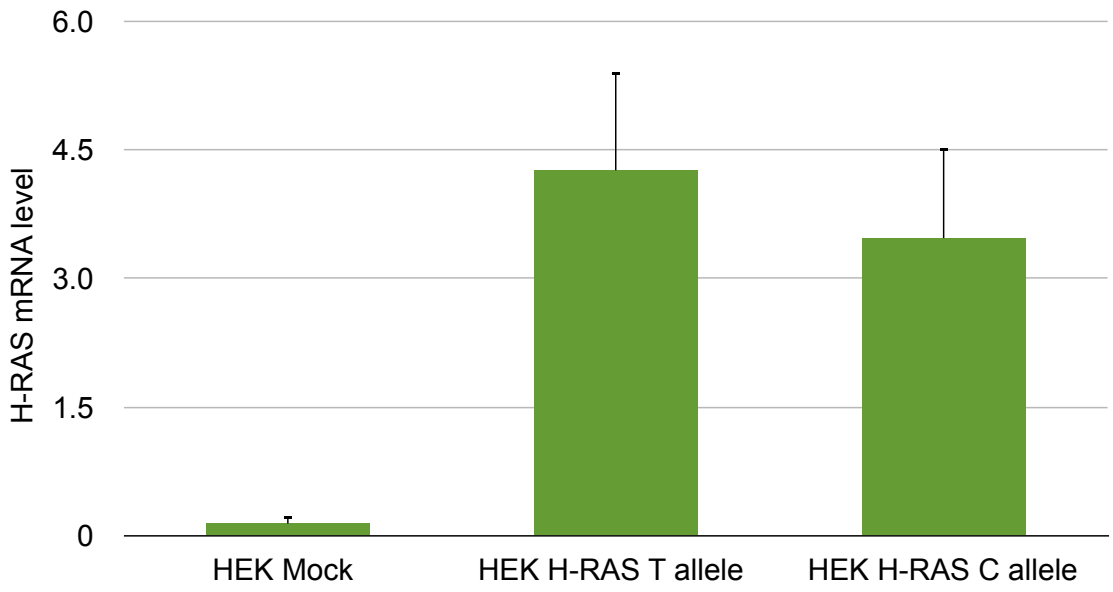
Figure 10 - Sequencing of the H-RAS in the HEK293 cells after transfection. A and B) Scheme of the H-RAS mini-gene. A) H-RAS mini-gene carrying the T allele in position 81. B) H-RAS mini-gene carrying the C allele in position 81. D and E) Electropherograms of the cDNA of the resulting cell-line: C) Mock cells; D) HEK293 transfected with the H-RAS carrying the T allele; E) HEK293 transfected with the H-RAS carrying the C allele.

The mRNA levels were evaluated by real-time RT-PCR, the HEK293 cells transfected with either version of the H-RAS mini-gene showed a significant increase of 30~ to 40~fold in the total amount of H-RAS mRNA (HEK293 cells carrying the T allele have a total amount of H-RAS mRNA of 4.26; HEK293 cell carrying the C allele have a total amount of H-RAS mRNA of 3.47), when compared with the HEK293 cells transfected with the empty vector (total amount of H-RAS mRNA is 0.14) (Figure 11A

and Table V). This result shows that the vectors carrying the H-RAS mini-gene are functioning properly, since the transfected cells have a significantly higher H-RAS mRNA expression than the cells transfected with the empty vector (HEK Mock) ($p<0.0001$).

We also verified if the H-RAS mini-gene were able to produce H-RAS protein, by Western blot, and we observed that the HEK293 cells carrying the T allele (p value=0.0036) and the C allele (p value=0.0009) express significant higher amounts of H-RAS protein, in comparison with the HEK293 cells transfected with the empty vector, but that the HEK293 cells carrying the T and C allele did not vary between them (p value=0.950) (Figure 11B, Figure 11C and Table V). The Western-blot was performed with an antibody that only recognizes the p21H-RAS, since there are no antibodies that recognize the p19H-RAS isoform.

A)



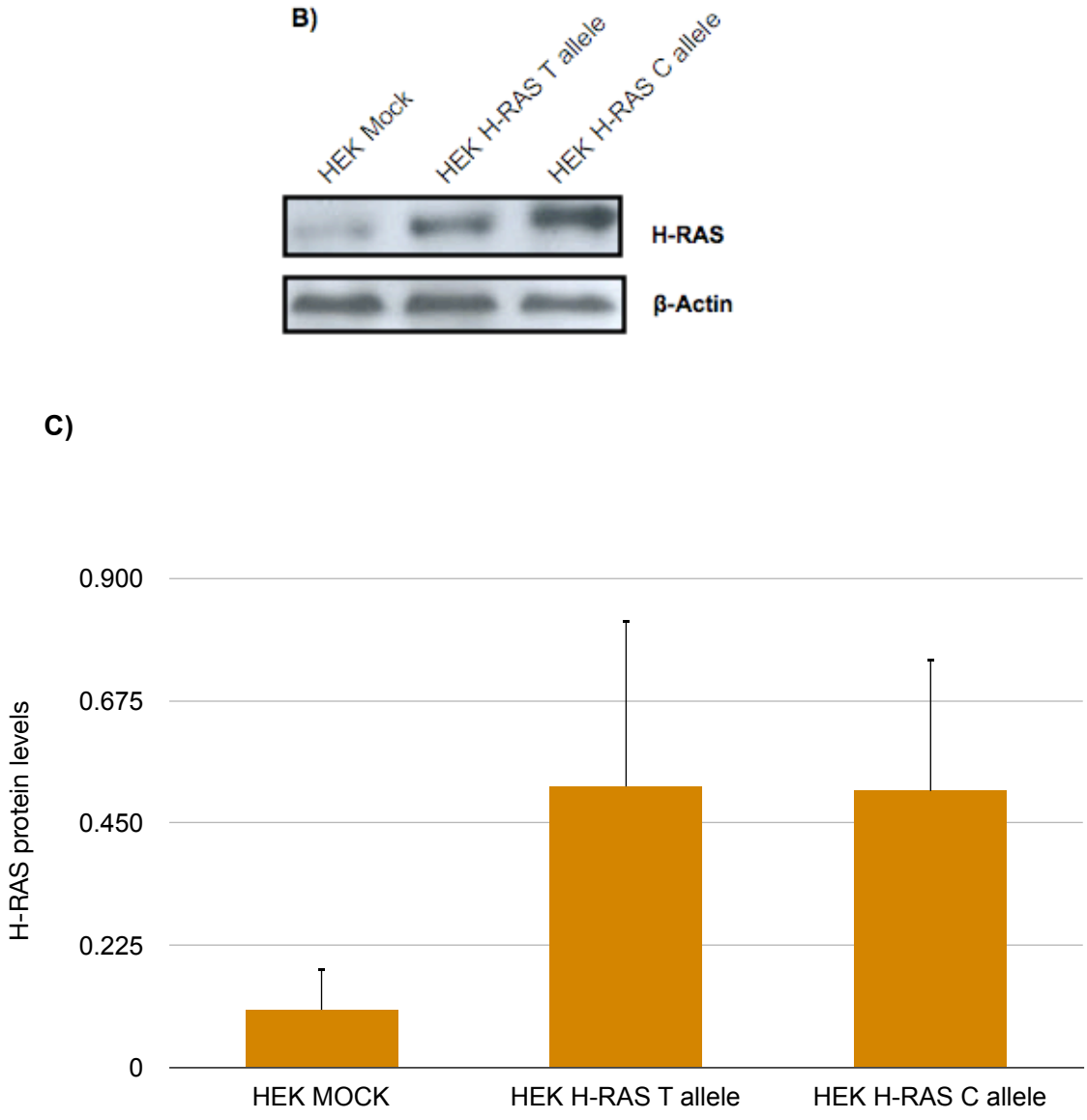


Figure 11 – H-RAS expression A) H-RAS mRNA expression in the HEK293 cells transfected with the empty vector (Mock) and with the H-RAS mini-gene, carrying the T allele or the C allele. The H-RAS expression was normalized with the GAPDH expression in each experiment. These are the results of three independent RNA extractions. B) Western-blot for the p21H-RAS. C) p21H-RAS protein expression quantification, normalized with α-tubulin. These are results from 3 replicas of 3 independent experiments.

Table V - Statistical analysis of the H-RAS expression, by the evaluation of the mRNA levels and protein expression.

	p value	
	mRNA expression	**Protein expression**
HEK Mock *vs* HEK H-RAS T allele	<0.0001	0.0036
HEK Mock vs HEK H-RAS C allele	<0.0001	0.0009
HEK H-RAS T allele *vs* HEK H-RAS C allele	0.025	0.95

Since one of the goals of the present study was to investigate if the presence of different H-RAS alleles could affect the splicing of the H-RAS gene, we measured, by real-time RT-PCR, the mRNA levels of the two H-RAS isoforms: p19H-RAS and p21H-RAS using validated probes from Applied Biosystems (Figure 12, Table VI), all the values were normalized with the expression of GAPDH.

The HEK293 cells transfected with the H-RAS genes showed a significantly increase of both the p21H-RAS (HEK293 cells carrying the T allele have an expression of 2.3; the HEK293 cells carrying the C allele have an expression of 2.15) and p19H-RAS isoforms (HEK293 cells carrying the T allele have an expression of 1.96; the HEK293 cells carrying the C allele have an expression of 1.32), when compared to the Mock cells (0.13 for the p21 isoform and 0.03 for the p19 isoform) (Figure 12), being this result concordant with the measurements of the total H-RAS mRNA. There are no differences in the amount of p21H-RAS between cells transfected with H-RAS carrying the T allele and the cells transfected with the H-RAS carrying the C allele (p value=0.68). The cells transfected with the H-RAS carrying the T allele have a slightly increase of the p19H-RAS isoform when compared to the cells transfected with the H-RAS carrying the C allele, but this difference does not reach statistically significance (p value=0.076) (Table VI).

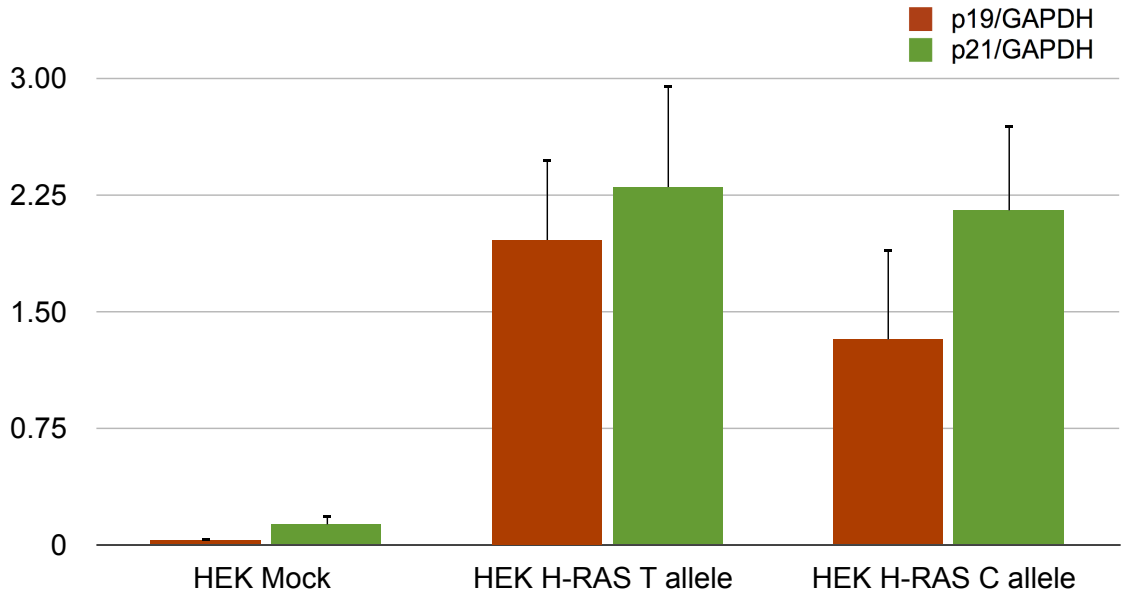


Figure 12 – mRNA expression in the HEK293 cells after transfection. Expression of the p19 and p21 H-RAS isoforms measured by real-time RT-PCR. All the values were normalized with the expression of GAPDH.

Table VI - Statistic analysis of the effect of the H-RAS 81T-C polymorphism in mRNA expression of the p19H-RAS and p21H-RAS.

	p value	
	p19H-RAS	**p21H-RAS**
HEK Mock *vs* HEK H-RAS T allele	<0.0001	<0.0001
HEK Mock vs HEK H-RAS C allele	<0.0001	0.0003
HEK H-RAS T allele *vs* HEK H-RAS C allele	0.076	0.68

With the type of transfection performed in this study we are not able to control the amount of integration of our vector in each transfection, therefore introducing an error when comparing the amount of the mRNA between different transfections (e.g. comparing the total amount of p19H-RAS between the HEK293 cells transfected with H-RAS carrying the T allele and the HEK293 cells transfected with H-RAS carrying the

C allele). For these reasons we decided that the most accurate measurement would be to use the expression ratio between the p19H-RAS and p21H-RAS isoforms.

The p21H-RAS/p19H-RAS ratio in the mock cells (p21H-RAS/p19H-RAS=4.333) is comparable to the values our group found in human tissues (Castro *et al*., 2006). In the cells transfected with the H-RAS mini-gene, there is a significantly decrease of the p21H-RAS/p19H-RAS ratio (p21H-RAS/p19H-RAS=1.173 for the HEK293 cells carrying the T allele; p21H-RAS/p19H-RAS=1.629 for the HEK293 cells carrying the C allele).

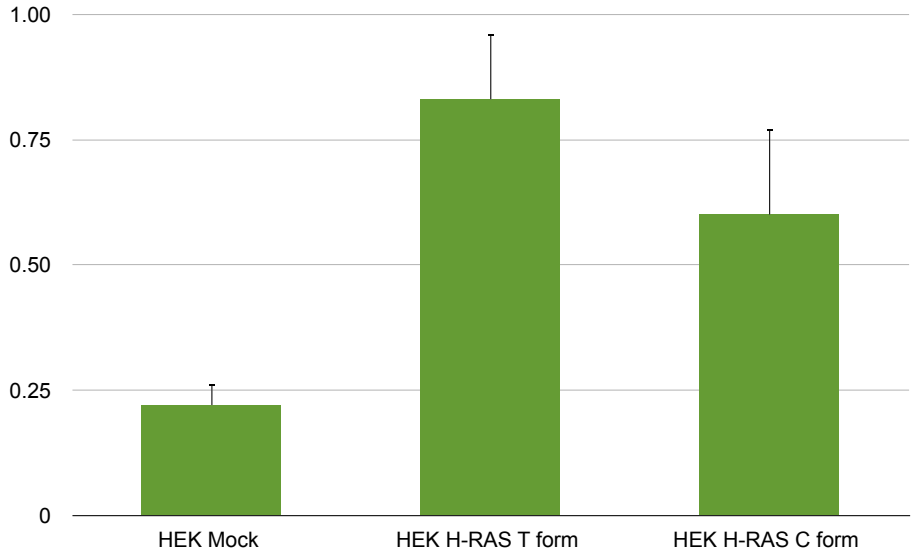


Figure 13 – mRNA p21H-RAS/p19H-RAS expression ratio in the HEK293 cells, after transfection.

Table VII - Statistic analysis of the mRNA expression of the p21H-RAS/p19H-RAS ratio.

	p value
HEK Mock *vs* HEK H-RAS T allele	<0.0001
HEK Mock vs HEK H-RAS C allele	0.0004
HEK H-RAS T allele *vs* HEK H-RAS C allele	0.03

The statistical analyses of the data showed that the HEK293 cells, transfected with H-RAS carrying the T allele, present a significantly lower ratio of the p21H-RAS/p19H-RAS, comparing with the cells transfected with H-RAS carrying the C allele (p=0.03) (Figure 13 and Table VII). Both cell-lines transfected with the H-RAS mini-gene (either carrying the T allele or the C allele) showed a significantly decrease of the p21H-RAS/p19H-RAS ratio.

3.2 – The H-RAS 81T-C polymorphism and aneuploidy

In an attempt to corroborate the previous results of our group, where we reported an association between the presence of the H-RAS 81C allele and aneuploidy in thyroid tumours, we analyzed the chromosome content of each cell-line transfected with the mini-genes. We performed chromosome counting of the metaphase spreads after an hypotonic treatment. The results of the chromosome counting in the metaphase spreads are summarized in the Table VIII and Figure 14.

Table VIII - Statistic analysis of the chromosome number in the cells transfected with the H-RAS mini-gene carrying 81T or 81C allele, in comparison with the cells carrying the empty vector (Mock).

	Chromosome number (mean)	Standard Deviation	p value	
HEK Mock (80 metaphases)	61.98	15.69		
HEK H-RAS T allele (79 metaphases)	65.73	18.60	P=0.17	P=0.0013
HEK H-RAS C allele (114 metaphases)	73.25	27.82	P=0.037	

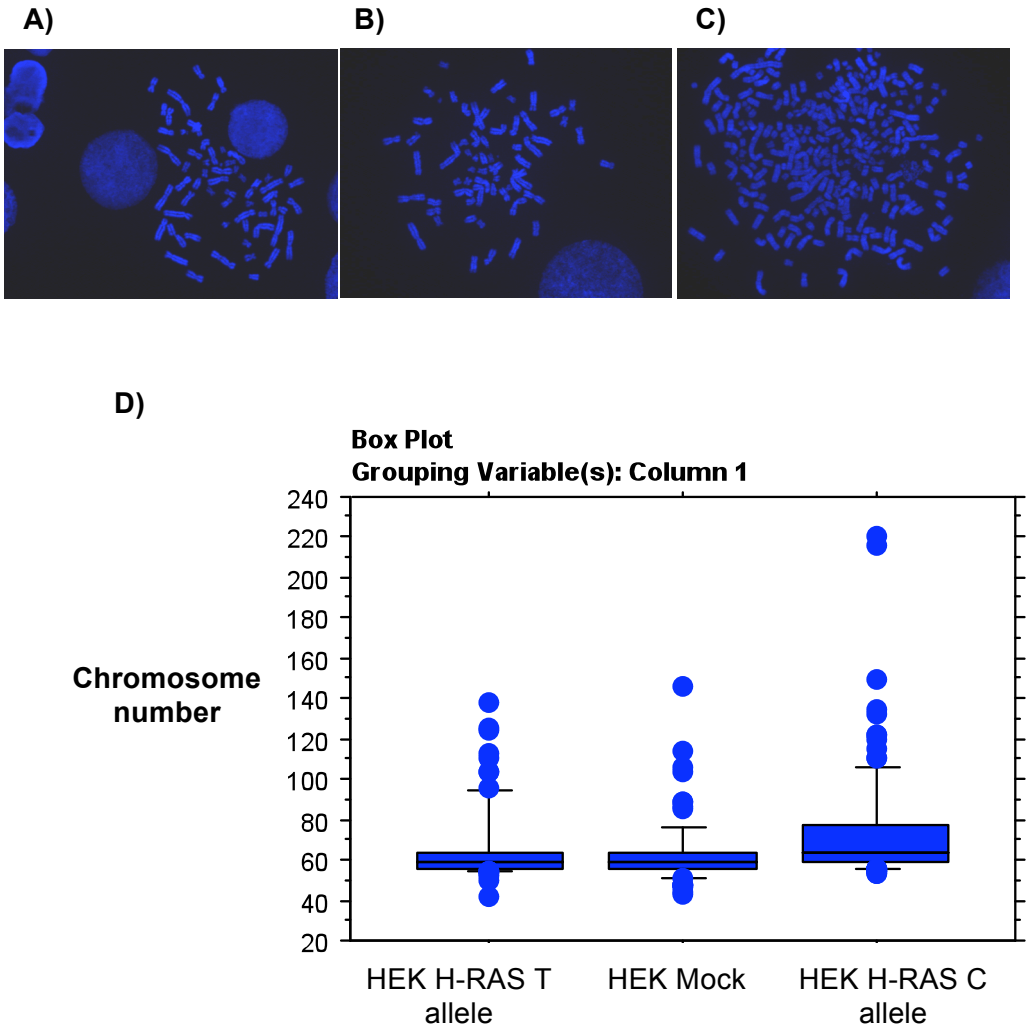


Figure 14 – Results of the chromosome spreads. A, B, C) Examples of chromosome spreads, stained with DAPI, after hypotonic treatment. A) HEK293 cells transfected with the empty vector (mock); B) HEK293 cells transfected with the H-RAS carrying the T allele; C) HEK293 cells transfected with the H-RAS carrying the C allele; D) Box plot analysis of the chromosome number in the 3 derived cell-lines. The middle line represents the mean chromosome number.

The mean chromosome number of the mock cells is 61.98±15.69, and is not significantly different from the mean chromosome number of the HEK293 cells transfected with the T allele, which is 65.73±18.6 (p value=0.17), meaning that transfection with the H-RAS gene carrying the T allele in the position 81 is not able to

induce chromosome instability. On the other hand, the HEK293 cells transfected with the C allele have a significantly higher mean chromosome number (73.25±27.82), than the mock cells (p value=0.0013) and the HEK293 cells transfected with the T allele (p value=0.0037). These results suggest that the C allele appears to induce aneuploidy in these cells. Another important finding is that the increased chromosomal instability found in the HEK293 cells transfected with the H-RAS carrying the C allele that may be indirectly seen by the higher standard deviation (Table VIII) harboured by the HEK293 cells transfected with the mini-gene carrying the C allele. Our results indicate that the HEK293 cells transfected with the C allele not only have a higher mean chromosome number but also show signs of more chromosomal instability, since there are more cells presenting a higher number of chromosomes than the mean chromosome number in the cells over-expressing the C allele.

Since the RAS oncogene has been associated with aneuploidy, through the MAPK kinase pathway, we decided to study the activity of this pathway, by studying the phosphorilation of the ERK proteins. We observe neither the cells carrying the T (p value=0.781) or the C allele (p value=0.912) present any significant difference when compared with the mock cells, or between them (p value=0.82), which indicates that the increased chromosomal instability present by the HEK293 cells carrying the C allele is independent from this pathway (Figure 15 and Table IX).

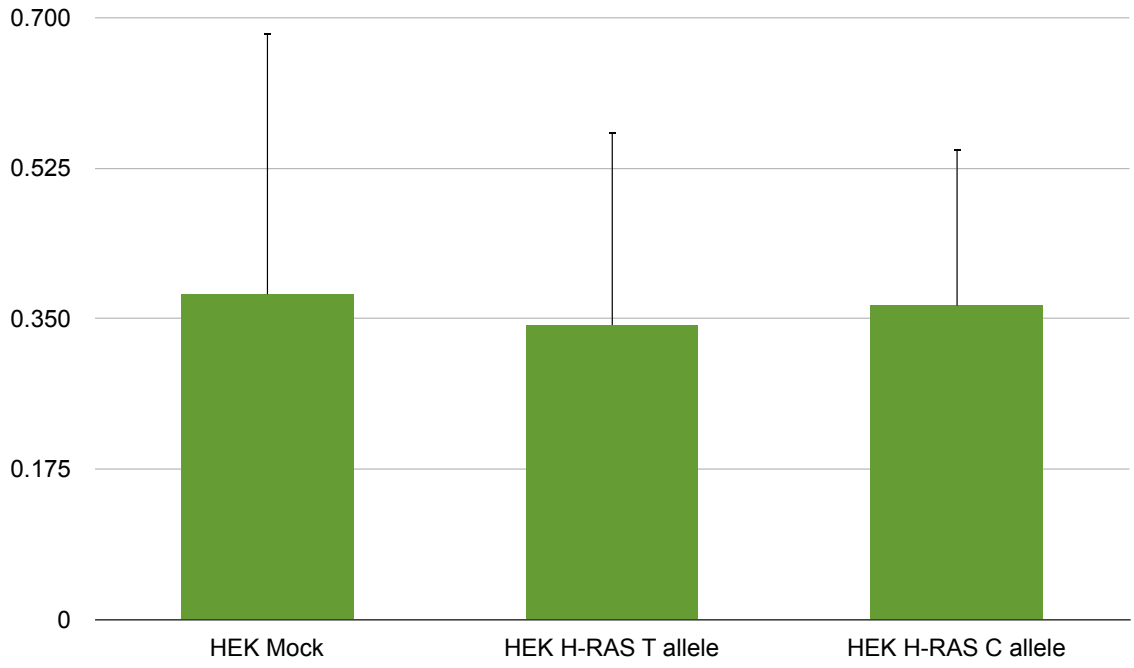


Figure 15 – PhosphoERK/ERK ratio by Western blot analysis. The quantified results were normalized with α-tubulin. These are results from 3 replicas of 3 independent experiments.

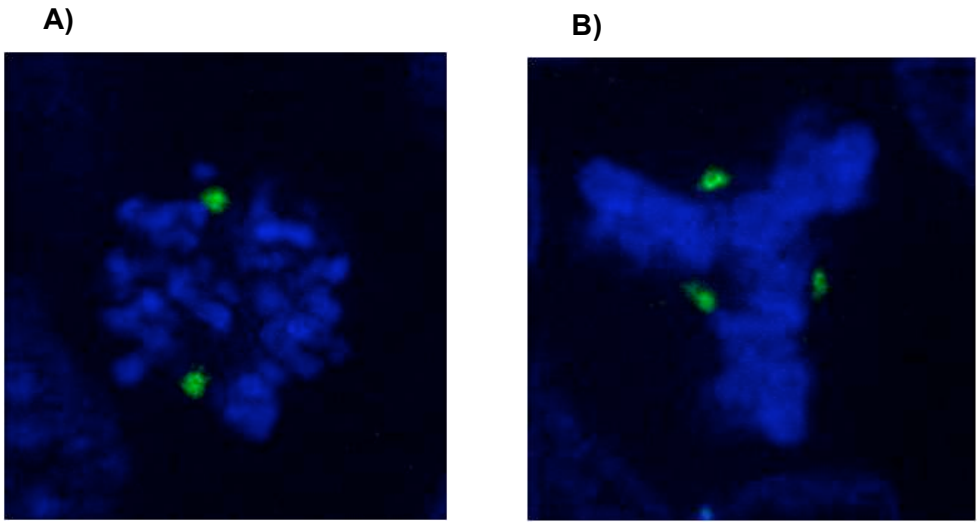
Table IX - Statistic analysis of the phosphoERKs/ERKs ratio.

	p value
HEK Mock *vs* HEK H-RAS T allele	0.781
HEK Mock vs HEK H-RAS C allele	0.912
HEK H-RAS T allele *vs* HEK H-RAS C allele	0.82

3.2.1 – The H-RAS 81T-C polymorphism and centrosome amplification

Centrosome amplification has been associated with aneuploidy, since the centrosomes are responsible for the bipolar spindle pole formation and for the cytokinesis. Several studies have shown that the RAS oncogene can lead to centrosome amplification, in thyroid cell lines and in mammary epithelial cells (Saavedra *et al*., 2000; Zeng *et al*., 2010). For this reason we decided to evaluate the

centrosome amplification by labelling the cells with pericentrin, which allowed us to count the centrosomes. Our results are summarized in the Figure 16 and Table X. The normal centrosome number in mitotic cells is two, to allow the cells to from a bipolar mitotic spindle. The mitotic cells over-expressing the C allele show a higher number of centrosome than the cells over-expressing the T allele or than the cells transfected with the empty vector (Mock) (Figure 16). This can be seen by the number of cells having a number of centrosomes higher than two: the mock cells have 3/100 (3%) cells, the cells transfected with the T allele have 5/117 (4.3%) cells and the cells transfected with the C allele have 29/165 (17.6%). Statistic analysis revealed that the differences between the cells transfected with the C allele and the mock and between the cells transfected with the C allele and the cells transfected with the T allele are statistically significant (p=0.0048 and p=0.0025, respectively) (Table X).



C)

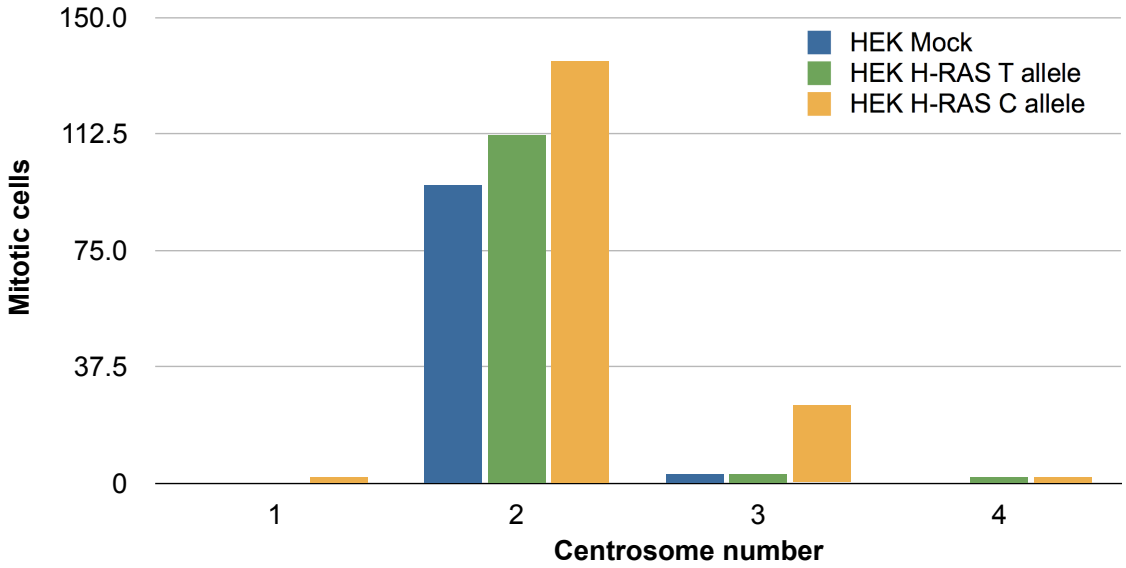


Figure 16 - Centrosome amplification in A) mitotic HEK293 cells carrying the T allele; B) mitotic HEK293 cells carrying the C allele. C) Chromosome amplification in mitotic HEK293 cells carrying the empty vector and carrying the mini-gene with the H-RAS 81 T or C allele.

Table X - Statistic analysis of the centrosome amplification.

	p value
HEK Mock *vs* HEK H-RAS T allele	0.41
HEK Mock vs HEK H-RAS C allele	0.0048
HEK H-RAS T allele *vs* HEK H-RAS C allele	0.0025

3.3 – The role of H-RAS 81T-C polymorphism in tumourigenesis

We also wanted to assess the effects of the H-RAS 81T-C polymorphism in the tumourigenicity of the HEK293 cells. As a first approach we decided to evaluate some classic features of cancer cells, such as the cellular growth, proliferation, apoptosis and migration.

For the evaluation of the cellular growth, we used the sulforhodamine B assay.

The C allele have a significant higher rate of growth, compared to the cells transfected with the H-RAS carrying the T (p=0.0223) allele and with the cells transfected with the empty vector (Mock) (p=0.0049) (Figure 17 and Table XI). We could not observe differences between the mock cells and the cells over-expressing the T allele (p=0.65).

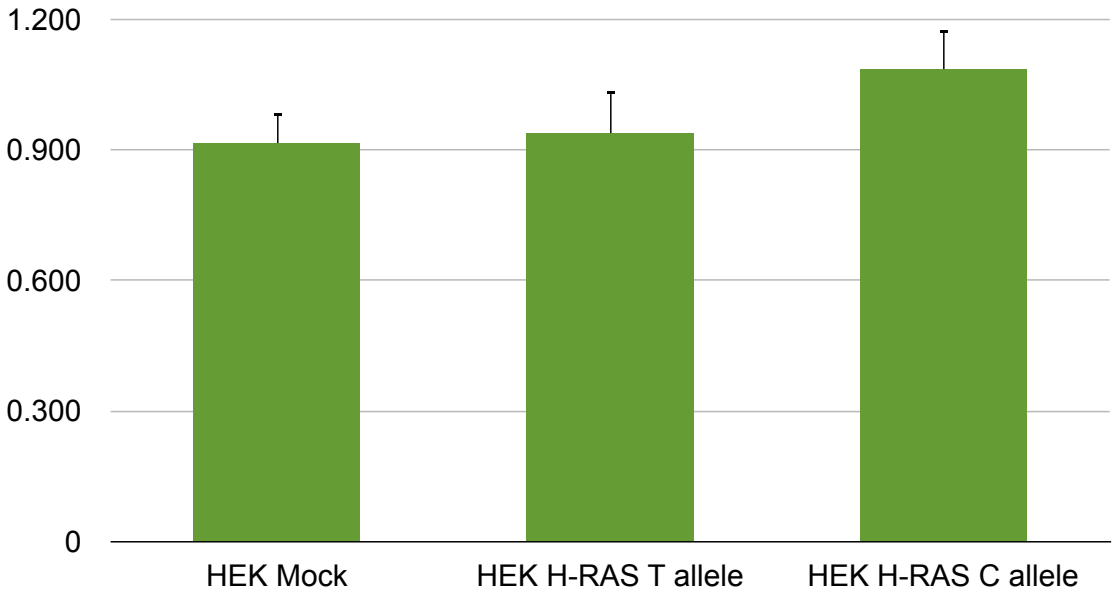


Figure 17 – Cell growth measured by the sulforhodamine B assay. These are results of 6 different experiments.

Table XI - Statistic analysis of the cellular growth of the transfected cells, measure by sulforhodamine B assay.

	p value
HEK Mock *vs* HEK H-RAS T allele	0.65
HEK Mock vs HEK H-RAS C allele	0.0049
HEK H-RAS T allele *vs* HEK H-RAS C allele	0.0223

The sulforhodamine B assay does not give any information in the reason for the differences in the growth of these cells. The differences observed in the growth of the

cells over-expressing the C allele could be due to an increase in cell proliferation and/or a decrease in cell death. We measured the effects of the H-RAS 81T-C polymorphism in the proliferation, by the BrDU proliferation assay. Our results show that, although the cells over-expressing the C allele have a trend to proliferate more, there are no significant differences between the different transfected cell lines (Figure 18 and Table XII).

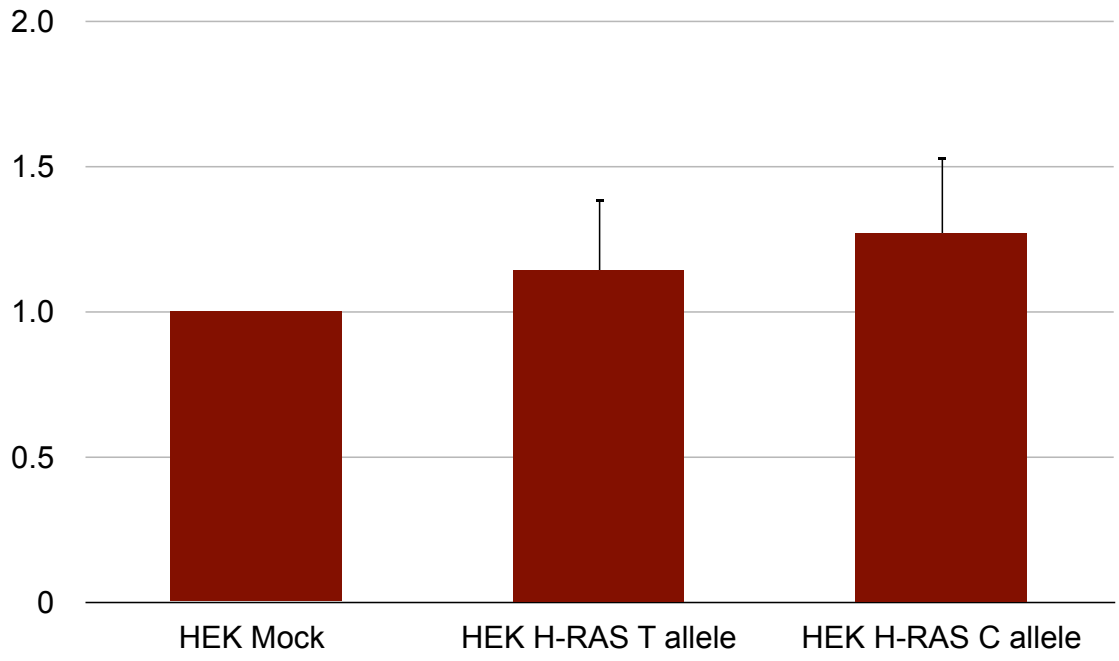


Figure 18 – Proliferation measured by the BrDU proliferation assay. These are results of 3 different experiments.

Table XII - Statistic analysis of the cellular proliferation of the transfected cells, measure by the BrDU proliferation assay.

	p value
HEK Mock *vs* HEK H-RAS T allele	0.3753
HEK Mock vs HEK H-RAS C allele	0.1525
HEK H-RAS T allele *vs* HEK H-RAS C allele	0.579

The other hypothesis was that the difference we observed in the growth could be due to differences in apoptosis. We analyzed cell death, by TUNEL assay. We verified that the HEK293 cells over-expressing the T allele have a significant higher rate of cell death than the HEK293 cells over-expressing the C allele (p value=0.0027) and that the HEK293 cells carrying the empty vector (p value=0.0063). We also observe that the HEK293 cells carrying the C allele have a significant decrease of cell death than the Mock cells (p value= 0.0035) (Figure 19 and Table XIII). These results corroborate the sulforhodamine B assay results.

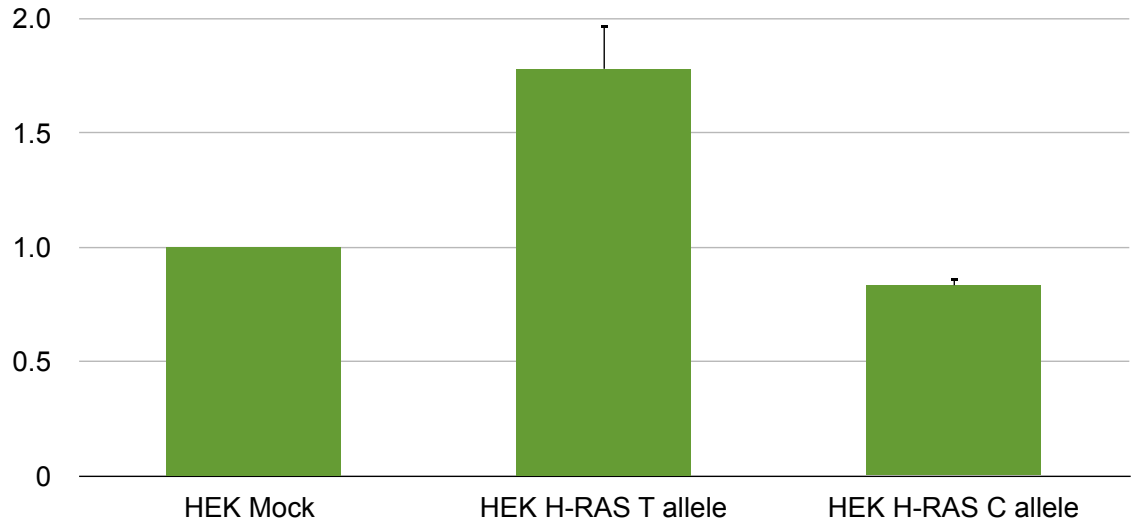


Figure 19 – Cell death accessed by TUNEL assay. These are results of 4 different experiments.

Table XIII - Statistic analysis of the cell death of the transfected cells, measure by the TUNEL assay.

	p value
HEK Mock *vs* HEK H-RAS T allele	0.0063
HEK Mock vs HEK H-RAS C allele	0.0035
HEK H-RAS T allele *vs* HEK H-RAS C allele	0.0027

One of the other main features of tumourigenic cells is the ability to be motile; therefore we wanted to evaluate the migration ability of the cells after transfection. For the migration study, we performed an in vitro wound-healing assay. We observed that the HEK293 cells over-expressing the C allele migrate significantly faster, in comparison with the HEK293 cells carrying the T allele (p value=0.0022 at 12h) and the HEK293 cells transfected with the empty vector (p value=0.0003 at 12h) (Figure 20 and Table XIV). We also showed that the HEK293 cells carrying the T allele also migrate significantly faster in comparison with the mock cells (p value=0.034 at 12h). This suggests, again, that H-RAS 81 C transfected cells display a more tumourigenic phenotype than the H-RAS 81T allele.

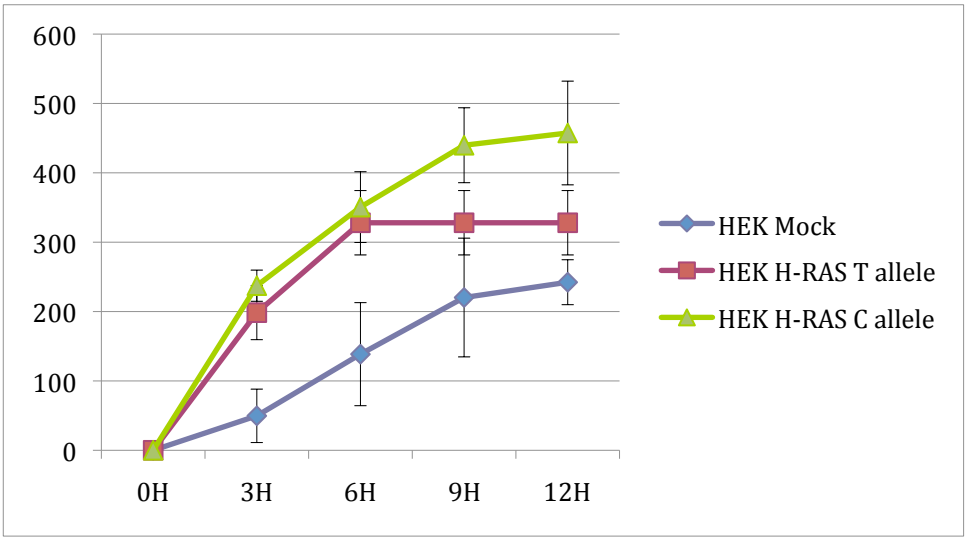


Figure 20 - Results of the wound-healing assay measured in 5 time-points. The scale is µm. These are the results of 3 independent experiments in each of which we performed 3 measurements.

Table XIV - Statistic analysis of the cell migration of the transfected cells, measure by the wound-healing assay.

	3h	6h	9h	15h
HEK Mock *vs* HEK H-RAS T allele	<0.0001	<0.0001	0.004	0.034
HEK Mock vs HEK H-RAS C allele	<0.0001	<0.0001	<0.0001	0.0003
HEK H-RAS T allele *vs* HEK H-RAS C allele	0.05	0.4	0.006	0.0022

Chapter 4 Discussion, Conclusions and Future perspectives

4.1 – Discussion

The H-RAS 81T-C polymorphism has been associated with an increased risk of some types of cancer: bladder cancer (Johne *et al*., 2003), oral cancer (Sathyan *et al*., 2006; Murugan *et al*., 2009), gastric cancer (Zhang *et al*., 2008) and colon cancer (Catela Ivkovic *et al*., 2009).

Our group did not find any association, in a Portuguese population, between the presence of the H-RAS 81T-C polymorphism and an increased risk of having thyroid tumours (Castro *et al*., 2006), but our group demonstrated that the C allele was associated with patients having aneuploid tumours (Castro *et al*., 2006).

This polymorphism does not lead to a change in the aminoacid in the protein, but Castro *et al* (2006) showed that the C allele was associated with an increase ratio of the p21/p19 in thyroid tumours and this could be a putative explanation for the aneuploid phenotype observed in the tumours.

With this work we aimed to establish a more reliable model to evaluate the role of the H-RAS 81T-C polymorphism in the cells, by transfecting the same cell-line (HEK293) with two versions of the H-RAS mini-gene (one carrying the T and the other carrying the C allele) and using the HEK293 cells transfected with the empty vector as controls.

4.1.1 – Effect of the 81T-C H-RAS polymorphism in the splicing of the H-RAS gene

Our first aim was to prove, experimentally, the previous observation about the association of the H-RAS 81T-C polymorphism and the splicing of the H-.RAS gene.

By transfecting the same cell line with two versions of the same H-RAS mini-gene, which only differs in the position 81 (either carrying a T or a C) gives us a straightfoward model to evaluate the effects of this polymorphism, and to exclude the

effect of any other regulatory sequences.

Using the real-time RT-PCR technique to quantify the mRNA levels of the H-RAS isoforms, we showed that the H-RAS 81T-C polymorphism leads to an alteration in the alternative splicing of the H-RAS gene. The HEK293 cells, transfected with H-RAS carrying the T allele, presented a significantly lower ratio of the p21H-RAS/p19H-RAS, comparing with the cells transfected with H-RAS carrying the C allele, which indicates that the H-RAS 81T-C polymorphism is involved in the alternative splicing of the H-RAS mRNA, being the T allele associated with a switch towards the p19H-RAS isoform and/or the C allele is associated with the p21H-RAS isoform (Figure 12 and Table VI). These results are in accordance to the study performed in thyroid tumours, where it was also observed an association between the H-RAS 81T-C polymorphism and an alteration of the p21H-RAS/p19H-RAS ratio (Castro *et al*., 2006). Although the mechanism associating this polymorphism with the alternative splicing variation remains unclear, our data suggests that this variation is due to the H-RAS 81T-C polymorphism itself and not by other sequence associated with it.

The significance of the difference in the p21H-RAS/p19H-RAS ratios in cells carrying the T or the C allele and the mock cells is still unclear, we showed that the cells transfected with either version of the H-RAS mini-gene has a significant lower p21H-RAS/p19H-RAS ratio than the mock cells (Figure 13 and Table VII). This could mean that the cells transfected with the H-RAS mini-genes would have a tremendous increase in the H-RAS expression (30 to 40 fold increase) and the cells would not be able to cope with such a high amount of the p21H-RAS without the p19H-RAS to counteract it. This hypothesis is particularly interesting, if one think that the p19H-RAS could be a negative regulator of the p21RAS (Huang and Cohen 1997). This hypothesis will need further studies. But the finding that the p19H-RAS is involved in the promotion of apoptosis (Jeong *et al*., 2006; Kim *et al*., 2008; Camats *et al*., 2009) delaying the G1/S phase (Camats *et al*., 2009) and diminish the glycolytic pathway in cancer and brain tissues, due to blockage of the NSE (Jang *et al*., 2009), are in the line

of reasoning that perhaps the p19H-RAS may antagonize the functions of p21RAS, like regulation of the cell cycle, promotion of cell proliferation and differentiation (reviewed in Karnoub and Weinberg 2008).

4.1.2 – Effects of the H-RAS 81T-C polymorphism in the chromosomal content

Taking into account the previous results published by our group about the association between patients carrying the H-RAS 81C allele and the presence of aneuploid tumours (Castro *et al*., 2006), we studied the effect of the H-RAS 81T-C polymorphism in the aneuploidization of the cell-lines after transfection, again taking advantage of having a clean model to evaluate the role of the polymorphism.

After performing the hypotonic treatment on the HEK293 cells (cells carrying the empty vector - mock, cells carrying the T allele and cells carrying the C allele) we counted the chromosomes in the metaphase spreads. The HEK293 cells are already aneuploid, as the majority of cultured cells, with a mean chromosome number of ≅75, which means that we can only observe the deviation from this number. We observed that the cells transfected with the mini-gene carrying the C allele have a significantly higher mean chromosome number than the mock cells (p value=0.037) and than the cells transfected with the T allele (p value=0.0013), showing that the C allele appears to be a “marker” of increased aneuploidy as previously shown by our group (Castro *et al*., 2006). We were, also, able to show that the HEK293 cells over-expressing the C allele were significantly more unstable than the HEK293 cells over-expressing the T allele or than the mock cells (Figure 14 and Table VIII), this is a very important results because it shows, for the first time, the importance of a polymorphism in the aneuploidization of the cells. One possible explanation for this fact is that HEK293 cells carrying the C allele have a higher p21H-RAS/p19H-RAS ratio than HEK293 cells carrying the T allele. This difference may indicate that the CIN and higher

chromosomal content in the cells carrying the C allele could be due to either an excess of 21H-RAS and/or reduced p19H-RAS, since the p21RAS oncogenic form as been associated with aneuploidy, through generation of abnormal chromosome content (Denko *et al*., 1994; Saavedra *et al*., 1999) and chromosome missegregation (Hagag *et al*., 1990; Saavedra *et al*., 1999); and the over-expression of the p21RAS have been shown to increase the tumourigenicity of the cells (Pulciani *et al*., 1985). One problem with this explanation is that the mock cells have a higher p21H-RAS/p19H-RAS ratio than the HEK293 cells carrying the C allele and the HEK293 cells carrying the T allele. One could argue that the mock cells are stable since they have the "endogenous" H-RAS expression, which is significantly lower than the HEK293 cells transfected with the H-RAS gene. Only when we increase the levels of the H-RAS expression the p21H-RAS/p19H-RAS ratio becomes crucial for the aneuploidization of the cells, since the amount of H-RAS that the cells need to cope is significantly higher. We cannot exclude the existence of other factors that can be influenced by the presence of the H-RAS 81T or 81C allele. We also excluded the hypothesis of the increased chromosomal instability could be due to the activation of the MAPK kinase pathway, reported a few years ago (Saavedra *et al* 1999), since our results does not present significant differences between the cells carrying the T allele, the C allele or the empty vector (Figure 15 and Table IX), but there are other proteins and pathways that can be activated by the RAS genes that we need to further investigate.

This is a very important finding, since it is the first time that a study links the H-RAS polymorphism and the p21H-RAS/p19H-RAS ratio with CIN. The link between the H-RAS and aneuploidy/CIN will be further investigated and the use of these cell lines will be a good model.

The data herein presented are consistent with the study of H-RAS 81T-C polymorphism in thyroid tumours, in which patients carrying the C allele have more often aneuploid tumours than patients homozygous for the T allele (Castro *et al*., 2006). The mechanism that leads to this event remains unclear, although it has been

suggested that it may be due to the higher ratio of p21H-RAS/p19H-RAS expression in the cases with the C allele (Castro *et al*., 2006). Our findings that the HEK293 cells transfected with the C allele have a higher p21H-RAS/p19H-RAS ratio than the HEK293 cells transfected with the T allele corroborate this hypothesis.

4.1.2.1 – Effects of the 81T-C H-RAS polymorphism in the centrosome amplification

Centrosome amplification has been associated with aneuploidy, as the centrosomes are responsible for the bipolar spindle pole formation and for the cytokinesis. The centrosome amplification will, therefore, affect severely these processes, leading to the formation of multipolar spindle poles and consequently chromosome missegregation (reviewed in Fukasawa 2005). In the past years, some studies have been emerging, stating that the RAS oncogene leads to centrosome amplification, in thyroid cell lines and in mammary epithelial cells (Saavedra *et al*., 2000; Zeng *et al*., 2010). We therefore evaluated the centrosome amplification, in the transfected HEK293 cells, by labelling the cells with pericentrin, which allowed us to count the centrosomes.

By counting the number of centrosomes in mitotic cells, we demonstrated that the cells over-expressing the C allele have a higher rate of centrosome amplification (Figure 16 and Table X), although the mechanism that gives rise to this amplification is yet to be elucidated. One of the hypotheses, that we excluded, was the failure of the cytokinesis. In the failure of the cytokinesis, the chromosomal content would double, due to the replication of the DNA in the S phase of the cell cycle and we do not observe a significant number of cells having the double chromosomal content (see above). Other hypothesis is related with the fact that the HEK293 cells carrying the C allele have a significant higher expression of the p21H-RAS protein. Several studies have stated that the over-expression of the normal p21RAS protein lead to an increase

in the tumourigenicity of the cells (Pulciani *et al*., 1985) and it is known that the RAS oncogene induces centrosome amplification and genomic instability (Saavedra *et al*., 2000; Zeng *et al*., 2010). So, our hypothesis is that the over-expression of the p21H-RAS in the HEK293 cells carrying the C allele leads to an increase of centrosome amplification.

4.1.3 – Evaluating the tumourigenic parameters in the HEK293 cells over-expressing each one of the H-RAS 81 alleles

Our final aim was to study the effect of the H-RAS 81T-C polymorphism in the tumourigenesis. The tumourigenicity of the cells can be evaluated by several features that are frequently altered in cancer cells, such as: the cellular growth, proliferation, apoptosis and migration.

To evaluate growth, we used the sulforhodamine B (SRB) assay. This method is used to determine cell density, by the binding of the sulforhodamine B dye to the protein content of the cells - it is a colorimetric assay to access cellular growth. Our results showed that this polymorphism affects significantly the growth of the cells, since the HEK293 cells over-expressing the C allele have a higher cell growth, in comparison with the HEK293 cells over-expressing the T allele (Figure 17 and Table XI). We also demonstrated that this difference in the cellular growth is due to an increased rate of apoptosis (Figure 19 and Table XIII) and not due to an increase in the cell proliferation (Figure 18 and Table XII). The increased rate of apoptosis can be caused by the over-expressing of the T allele, which may lead to decrease in the p21H-RAS/p19H-RAS ratio in the cells transfected with the mini-gene carrying the T allele. This result is in accordance to previous published data, since the p19H-RAS has been reported to induce apoptosis, through the p73β pathway (Jeong *et al*., 2006; Kim *et al*., 2008). These results will be explored by checking the levels of the proteins of the p73β pathway.

Other important feature evaluated was the cell migration, by the wound healing assay. With this experiment we were able to show that the two cell-lines over-expressing the H-RAS mini-genes migrate significantly faster than the mock cells (Figure 20 and Table XIV), which can be explained by the higher amount of the oncogene that is known to promote cell migration (Bian *et al*., 2004; Shin *et al*., 2005). We also demonstrated that the cells over-expressing the C allele migrate significantly faster than the cells over-expressing the T allele (Figure 20 and Table XIV), which can be due to the higher expression of p21H-RAS/p19H-RAS in the cells carrying the C allele, since the p21RAS proteins are the known effectors in the signalling pathways that promote cell migration (Simpson *et al*., 2008; Drosten *et al*., 2010) and the oncogenic form of the p21RAS leads to an increase in the cell migration (Bian *et al*., 2004; Shin *et al*., 2005).

4.2 – Conclusions

The main conclusion of the present study was that a well-known oncogene, the H-RAS, has a frequent polymorphism - 81T-C polymorphism – that has measurable effects in the behaviour of the cells. Whether or not all the studied effects of the H-RAS 81T-C polymorphism are due to an alteration of the p21H-RAS/p19H-RAS ratio remains to be elucidated.

. Our results have demonstrated that the H-RAS 81T-C polymorphism itself is associated with an altered splicing of the H-RAS gene. How this alternative splicing is influenced by the 81T-C polymorphism and if there are other proteins involved needs to be further studied. We hypothesized that the alteration in the isoforms expression is the reason why the H-RAS 81C allele displays a “more oncogenic” effect than the H-RAS 81T allele. This assumption is in accordance with the reports stating that the p19H-RAS behaves more like a tumour-suppressor than like an oncogene (Huang and Cohen 1997). We demonstrated that the H-RAS 81C allele presented an increased

chromosomal instability, increased cell growth, decreased cell death and increased cell migration, in comparison with the mock and/or with the cells over-expressing the T allele. The relationship between the polymorphism and aneuploidy will be further investigated to understand the mechanisms and to find in which part of the cell-cycle is the problem occurring. The phenotype caused by the cells over-expressing the C allele can be, partially, justified by the fact that this polymorphism can affect the splicing of the H-RAS gene, as seen in real-time results, being the C allele associated with a higher ratio of p21H-RAS/p19H-RAS than the T allele.

4.3 – Future perspectives

Since this is the first time that a study associates the H-RAS 81T-C polymorphism with chromosomal instability, we intend to uncover the mechanism responsible for this, by studying the p19H-RAS isoform, through p19H-RAS over-expression (using a vector carrying the H-RAS gene lacking exon 4) and through the silencing of p19RAS (using short hairpin RNA targeting the IDX exon). Using this strategy we will understand if it is the ratio of the p21/p19 H-RAS isoforms that is responsible for the phenotypes observed in the transfected HEK293.

We also intend to study better the cell cycle regulation of the HEK293 cells transfected with the H-RAS mini-gene, carrying the T allele or carrying the C allele, in order to reveal possible alterations that lead to chromosome missegregation and, consequently, aneuploidy. To achieve this aim, we will follow the cells in mitosis to check where are the errors occurring, to decide, afterwards, which will be the pathways to study in depth.

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